THE RELATIONSHIP BETWEEN THE EFFECTS OF HIGH TEMPERATURE ON CHLOROPLAST MEMBRANE INTEGRITY AND CHLOROPLAST LIPID COMPOSITION

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

NOMENCLATURE

SL	Sulfolipid
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PG	Phosphatidylglycerol
DPG	Diphosphatidylglycerol
MGDG	Monogalactodigylceride
DGDG	Digalactodiglyceride
PC	Phosphatidylcholine
PL	Phospholipid
PS	Phosphatidylserine

CHAPTER I

INTRODUCTION

Temperature is one of the most important environmental factors affecting the living organism. Living organisms have the capacity to grow and maintain metabolic activity between $-2^{o}C$ and $100^{o}C$, a range sometimes referred to as the "biokinetic zone." However, individual organisms have much narrower temperature limits. Life as we know it developed in an aqueous environment; the requirement for water plays a highly important role in restricting these temperature limits.

One of the earliest observations concerning temperature limits for individual organisms was that some organisms can maintain a relatively constant body temperature over a wide range of environmental temperature and are referred to as homeotherms. Plants, Monera, Fungi, Protists and the lower orders of animals, however, do not have this capacity. Their body temperature is largely determined by the environmental temperature. These organisms are called poikilotherms. Some poikilotherms can survive over a wide thermal range and are referred to as eurythermal organisms while others called stenothermal organisms have a much more narrow range of temperature tolerance.

The effects of supraoptimal temperatures have been reviewed by Levitt (1972), Rose (1967), Alexandrov (1964), and others. In the initial stages of heat injury many of the effects are reversible but will lead to "thermal death" if continued. Accumulative heat injury

eventually results in total inhibition of the vital functions of plants (Daniell et al., 1969). In 1964, Alexandrov used such criteria as suppression of plasmolysis and deplasmolysis, exit of pigments from the vacuoles, loss of vital staining, luminescence of chloroplasts, and the cessation of protoplasmic streaming to determine the injury to cells from exposure to supraoptimal temperatures.

The specific physiological or biochemical changes which initiate cell death are still unknown. In 1957, Petinov and Molotkovsky suggested that indirect heat injury is due to the toxic effect of NH_3 produced at high temperatures and that this effect is counteracted by the production of organic acids (see Levitt, 1972). This idea may help to explain the heat tolerance of succulents which are CAM plants. A second possibility is the production of biochemical lesions (Kurtz, 1958; Neales, 1968; Starr and Parks, 1962; and others). In 1963, Al'tergot suggested that these lesions might be due to the thermostability of oxidases which, as temperature increases, destroy such oxidizable substances as ascorbic acid and glutathione producing biochemical lesions. Lepeschkin (1935) presented evidence suggesting that heat injury may result from a net breakdown in protoplasmic proteins (see Levitt, 1972). This decrease in protein may result from decreased synthesis or increases breakdown. Some strains of Neurospora crassa are able to produce the hydrolytic enzyme cellulase in the presence of cellulose at 35°C but fail to product this enzyme at 25°C (Hirsch, 1954). It is possible that thermal injury is due to a self-digesting proteolytic enzyme produced at high temperature, possibly by lysosomes (Levitt, 1972). That the loss in protein was caused by decreased synthesis was supported by Schiebel et al. (1969) and others. Alexandrov (1964)

produced evidence of repair mechanisms. Also, "hardening" can increase the thermostability of proteins (Feldman, 1966).

These effects of heat injury described so far are primarily indirect damage requiring long periods of exposure. Direct injury is induced by short exposure with damage occurring during or immediately after exposure (Levitt, 1972). There are three important hypotheses explaining the mechanism(s) of direct heat damage to plant cells.

Belehradek (1935) first explained heat injury on the basis of protein denaturation. In 1912, Lepeschkin stated that all layers of the protoplasm, including the plasma membrane, coagulate simultaneously at the heat-killing temperature. He therefore determined protoplasmic coagulation by the time semipermeability of the plasma membrane was lost, i.e., when the pigments of the cell sap were observed to diffuse out under the microscope (see Levitt, 1972).

Heilbrunn suggested that high temperatures initiated the liquification of protoplasmic lipids (see Levitt, 1972). Attempts to support this idea were unsuccessful (Belehradek, 1935; Campbell and Pace, 1968). Lepeschkin (1935) cited evidence suggesting that liquification of protoplasmic lipids occurs as a result of death, not as the cause. However, Kleinschmidt and McMahon (1970a, 1970b) presented evidence that growth temperature has a direct effect on the ratio of saturated to unsaturated fatty acids of the membrane lipids. Loss of membrane semipermeability at high temperatures could therefore be due to changes in membrane fluidity producing loss of membrane integrity.

Attempts have also been made to implicate denaturation of nucleic acids by heat as the direct cause of cell death, but there is little evidence to support this idea (Levitt, 1972).

Levitt (1972) stated that the most likely cause of heat injury was denaturation of plant proteins and the subsequent inactivation of protein synthesizing enzymes. Here, direct heat injury is most likely due to denaturation of membrane proteins causing loss of differential membrane permeability and cell death. Alexandrov (1956, 1965) stated that the thermostability of living cells was determined by the thermostability of their proteins (see Levitt, 1966). However, Maximov (1938) noted that the action of supraoptimal temperatures as explained by the protein theory was often inadequate since death often occurred at a temperature below the denaturation point of proteins.

The limits of temperature at which organisms can survive might be related to membrane stability (Daniell et al., 1969). Brock (1967) suggested that thermal death was a first order process implying that a single event within the cell initiated this response. The enzymes within the living cell comprise an extremely complex system with varied levels of thermal stability. Although it was possible that a single enzyme could initiate thermal death, it was unlikely that it would result in immediate death of the cell. It seemed more likely that thermal death would result from disruption of cellular membranes whose integrity was essential for cell function. This may also help explain why eucaryotes were more thermal sensitive than procaryotes (Klienschmidt and McMahon, 1970b).

Pearcy et al. (1977) cited evidence pointing toward photosynthesis as the most thermal sensitive process in cellular metabolism. Photosynthesis was found to decrease at temperatures several degrees below the temperatures at which protoplasmic streaming, respiration and other metabolic processes were affected.

Santarius (1975) compared the effects of heat on four different chloroplast stroma enzymes with photosystem II dependent electron transport and found that the enzymes were inactivated at much higher temperatures than the photosynthetic process, suggesting that the chloroplast membranes were the most thermal sensitive component of the photosynthetic apparatus, agreeing with Daniell et al. (1969).

The photosynthetic apparatus of many plant species is capable of physiological acclimation to changes in prevailing temperatures. These changes were expressed in improved photosynthetic performance. Pearcy (1978) suggested that thermally induced changes in chloroplast lipids were significantly related to the greater thermal stability of the photosynthetic apparatus at higher growth temperatures.

CHAPTER II

LITERATURE REVIEW

General Membrane Structure

Danielli and Davson (1935) visualized the membrane as a lipid bilayer wherein lipids are lined up side by side with their polar (hydrophobic) groups facing inward, and with globular proteins attached symmetrically on either side of the lipid bilayer. Robertson (1959) proposed a membrane model where the proteins were spread out over the lipid bilayer rather than in globular form. This model was found to be thermodynamically unstable since it exposed the non-polar (hydrophobic) amino acids of the proteins to water and covered the polar and ionic (hydrophilic) head groups of the lipids (Quinn, 1976).

Singer (1971) and Singer and Nicholson (1972) developed a concept of membrane structure to explain the highly mobile character of individual membrane lipids. Circular dichroism data indicated that the bulk of the membrane polypeptides are in the α-helix configuration suggesting that the membrane proteins are primarily globular rather than stretched out in a monolayer (Singer, 1974). Singer (1971) and Singer and Nicholson (1972) concluded that cell membranes consist of a lipid bilayer matrix into which the proteins are imbedded. Some proteins are probably imbedded in the upper lipid layer, some in the lower lipid layer, and some probably extend all the way through the lipid bilayer.

Like lipids, proteins are amphipathic with hydrophilic colar, ionic groups projecting out into the aqueous exterior and hydrophobic non-polar groups imbedded among the hydrocarbons of the membrane lipids. The apparently random distribution of the proteins among the lipids suggests a mosiac pattern. Freeze fracture and spin label studies also suggested that membrane lipid diffuse rapidly in the plane of biological membranes indicating a fluid rather than a rigid lipid bilayer. Although the redistribution of membrane particles is more easily explained by lipid rearrangement, one cannot entirely exclude membrane protein migration (Quinn, 1976).

Protein distribution within the membrane is maintained by thermal motion and charge repulsion between ionized polar groups. This motion is reflected in a rapid rotation of protein molecules about an axis perpendicular to the plane of the membrane and at a rate dependent upon the fluidity of the membrane lipids. The lateral mobility of membrane proteins is evident from their ability to aggregate when electrostatic repulsion between them is destroyed or when their binding sites are complexed by antibodies. The speed of movement of species--specific antigens across a cell membrane is dependent upon the fluidity of the membrane lipids and does not require metabolic energy (Quinn, 1976).

The dynamic structure of membranes can be exemplified by the observation that individual membrane components can be inserted and removed from one location to another without destroying--or significantly disturbing--the overall structure of the membrane.

Nearly all cells can synthesize all of the membrane components necessary for their growth and development. The major site of membrane lipid and protein synthesis is the endoplasmic reticulum. Membrane

proteins and lipids have been found to turn over at different rates within membranes with phospholipids--especially phosphatidyl inositol and phosphatidic acid--turning over faster than membrane proteins. These phospholipids can be exchanged between cell membrane systems and the endoplasmic reticulum by specific exchange proteins. There is evidence that a given membrane varies in composition and permeability properties as the cell matures and in response to environmental variations.

The hydrocarbon chains of membrane lipids can exist in two temperature dependent forms. An ordered crystalline phase occurs at lower temperature and a disordered liquid crystalline phase exists at higher temperature. The particular phase at any temperature depends on the length and the degree of saturation of the hydrocarbon chain and depends to some extent on the nature of the polar interaction with water. Membrane lipids adapted to lower temperatures have higher levels of unsaturation of their fatty acids while those adapted to higher temperatures have more saturated fatty acids.

Selective permeability is one of the most important physiological properties of biological membranes. Membrane permeability is an expression of the freedom of movement through them. Cell membranes are relatively permeable to gases and water mainly because of their small size. Some membrane proteins have a subunit structure which sometimes contains narrow, water-filled channels near their central axis through which water and other small molecules could pass.

Interaction between lipids and proteins within membranes could act as a barrier to the movement of materials through either or both

membrane constituents. Modifications of either or both constituents could alter the permeability properties of the membrane.

Bretscher (1973) and Zwaal (1973) suggested an asymmetric distribution of lipids between the inner and outer layers of membrane lipids in erythrocytes. Anderson (1975b) suggested that this may be a general characteristic of all biological membranes. Jost et al. (1973) and Dehlinger et al. (1974) suggested further differentiation along membranes with boundary lipid fixed in position surrounding the hydrophobic regions of membrane proteins while more fluid lipids are positioned in the matrix of the fluid bilayer. Although there is no firm evidence for pinpointing the position of any lipid species in the membrane, Anderson (1975) suggested that one could predict where various classes of lipids might be found in the lipid-protein mosaic model from the composition and properties of each lipid class.

Nearly all biological membranes possess some form of enzyme activity. Some of these enzymes are integral parts of the membrane structure. Many of these membrane enzymes are found exclusively in one type of membrane which provides a useful marker for that particular membrane (Brown and Chattopadhyay, 1971; Pierre and Karnovsky, 1973). Rothfield and Romeo (1971) and Griffith et al. (1973) demonstrated the requirement for specific lipid halos for specific membrane-bound enzymes to function. Although the specific nature of these halos is not understood, Rothfied and Romeo (1971) and Tsukagoshi (1973) suggested that a specific lipid-requiring enzyme could be inserted into that part of the membrane with the proper lipid composition or perhaps complexed with specific lipids prior to insertion into the membrane.

The first possibility would suggest a patchwork distribution of lipids in the membrane rather than random homogeneous lipid distribution.

Lipid Composition of Chloroplasts

In 1938, Menke (see Lichtenthaler and Park, 1963) separated chloroplasts from spinach leaves, using ammonium sulfate fractionation. Chemical analysis indicated that the chloroplasts contained 53% protein and 31% lipids and that most of the leaf lipids were located in the chloroplasts. Menke published a second paper later that same year using chloroplasts isolated by centrifugation and found 48% protein and 37% lipids. Variations in composition were attributed to cytoplasmic contamination of the chloroplasts. In 1942, Menke and Jacob separated spinach chloroplast lipids but did not bother with cytoplasmic contamination since they did not consider them a significant contaminant. Zill and Harmon (1962) found that as much as one-third of the whole leaf lipids are not present in the chloroplasts which cast doubt on the results of Menke and Jacobs. In 1942, Bot (see Lichtenthaler and Park, 1963) investigated the chemical composition of spinach grana isolated by centrifugation and found that they contained 42-54% protein and 26-32% lipids which seemed to vary with the season of the year and age. In 1962, Weber (see Lichtenthaler and Park, 1963) reported a lipid to protein ratio of 0.7-1. Park and Pon (1963) found that spinach chloroplasts have a lipid to protein ratio of approximately 1.0 as compared to a ratio of 0.6 reported by Bot (1942). However, Bot's protein may have been a little high due to

grana contamination. There is also evidence that Bot's lipid determinations were low due to incomplete extraction. Park and Pon (1961, 1963) were able to purify chloroplast lamellae which were active in the Hill reaction and consistently yielded a lipid to protein ratio of 1.0.

Ferrari and Benson (1961) analyzed the lipid composition of algae and higher plant photosynthetic tissue with general agreement that galactolipids (GL) are most abundant with lesser amounts of phospholipids and sulfoquinovosyldiglyceride.

Winterman (1960) analyzed the phospholipid composition of chloroplast lipids and reported 53 moles phospholipid per 105 moles of chlorophyll. The distribution of phospholipids was found to be 6 moles phosphatidyl inositol (PI), 24 moles phosphatidyl glycerol (PG), 3 moles phosphatidyl ethanolamine (PE), 19 moles phosphatidyl choline (PC), and 1 mole phosphatidic acid (PA). In 1962, Winterman (see Lichtenthaler and Park, 1963) reported that monogalactosyldiglyceride (MGDG) and digalactosydiyceride (DGDG) accounted for 158 moles and 66 moles per 105 moles of chlorophyll respectively plus 22 moles of sulfoquinosyldiglyceride (SL) per 105 moles of chlorophyll. Winterman's results were supported by Zill and Harmon (1962) who reported that MGDG, DGDG, PL, and SL represented the vast majority of spinach chloroplast lipids.

In 1966, Allen et al. presented a comprehensive lipid analysis of spinach leaf and chloroplast lamallae. Results indicated that of 123 mg of material identified as lipid, 47 mg were MGDG, 32 mg were DGDG, 17 mg were PG, 14 mg SL, 6 mg PC, 4 mg unknown, and 3 mg PI. Allen et al. (1966) indicated that 48.5% of lamellar dry weight is lipid as

compared to 55% by Park and Pon (1963). Appreciable differences, however, are to be expected with different starting material, age, and environmental factors (Allen et al., 1966).

Roughan and Batt (1969) isolated photosynthetic leaf lipids from twenty different plants including algae, liverwarts, mosses, Gingko, Pinus and numerous Angiosperms, both dicots and monocots. Mono- and digalactodiglycerides were the most abundant lipids in that order in all cases with small quantities of sulfolipid and phospholipids. Variability between these plants varied even less when calculated relative to chlorophyll content rather than fresh weight of photosynthetic tissue.

Schwertner and Biale (1973), using intact chloroplasts isolated from avocado fruit and cauliflower leaves, separates and quantitatively determined their lipid composition. The amounts of galactolipids and phospholipids present in cauliflower leaf chloroplast were similar to those reported for spinach lamellae by Allen et al. (1966) and for tobacco leaves by Ongun et al. (1968) while the lipid composition of avocado was quite different with significantly higher quantities by weight of phospholipids (20.4% to 9.1%) with major decreases in monogalactosyldiglyceride (14.4% to 9%).

MacKender and Leech (1974) attempted to isolate Vicia faba L. var. dwarf white fan leaf chloroplasts for chemical characterization of the chloroplast envelope membrane lipids as compared to lamellar lipids. Results of these analyses indicated that the acyl lipids of both types of chloroplast membranes are qualitatively identical but their proportions are quite different. Quantitative analysis and comparison of lipid species in chloroplast envelopes compared to

chloroplast lamellae indicated 29.1% to 65.4% monogalactosyldiglyceride, 32.4% to 26.2% digalactosyldiglyceride, 29.6% to 2.8% phosphatidylcholine, and 8.9% to 5.5% phosphatidylglycerol respectively. This data reflected a MGDG/DGDG ratio of 0.9:1.0 in the envelope and 2.4:1 in the lamellae. Total galactolipid represented 61.5% of total lipids in chloroplast envelopes and 91.6% in lamellae. Although phosphatidyl choline and phosphatidyl glycerol were shown to be the major phospholipids in both membranes, phosphatidylcholine was shown to represent 29.6% of the total chloroplast envelope lipid but only 2.8% of the lamellar lipids. MacKender and Leech (1974) omitted the sulfolipid from their data as a major membrane lipid although they did mention it as a minor component.

Poincelot (1973) compared the lipid composition of undifferenaited chloroplasts, mesophyll chloroplasts, and bundle sheath chloroplast fragments of maize. Results indicated no significant qualitative differences between these groups. However, quantitative differences were observed. The most significant changes are expressed in terms of MGDG/DGDG ratio between undifferentiated and mosphyll chloroplasts which were found to be 1.08: and 2.48:1, respectively. There was, however, little if any change in sulfolipid or phospholipid concentrations as the chloroplasts matured. Changes in lipid concentration between undifferentiated chloroplasts and mesophyll chloroplasts were 4.1% to 5.2% SL, 3.9% to 4.1% PG, 0.5% to 0.6% PC, and 0.1% to 0.4% DPG, respectively. An interesting fact here was the close similarity between maize mesophyll chloroplast lipid composition and the less efficient photosynthetic species like spinach (Kates, 1970), tobacco and sugar beet (Hitchcock and Nichols, 1971).

Fatty acid analysis of whole spinach leaves indicated that linoleic acid is the major component with 34.7% of the total with oleic (26.3%) and linolenic (12.7%) present in significant amounts. Much of the fatty acid present existed not as part of lipids but as free fatty acids. More recently, Wolf et al. (1962) and Debuch (1962) determined the fatty acid content of isolated spinach chloroplasts using gas liquid chromatography as 68.9% and 47.8% linolenic, 10.8% and 19.5% in 16:3 fatty acid (unidentified), 11.2% and 15.5% palmitic, 4.6% and 5.0% linoleic, respectively, with trace amounts of oleic and other fatty acids. Although relatively accurate determination of fatty acids present was made, no distinction was made between free and esterfied fatty acid, a very important factor.

Comprehensive lipid analysis by Allen et al. (1966) illustrated the fatty acid composition of each spinach chloroplast lamella lipid species. Linolenic acid (18:3) was found to be the major component of monogalacto- and digalactosyldiglyceride with 71.9% and 86.7%, respectively. A hexadecatrienoic acid (16:3) was also quite high in monogalactodiglyceride (25%) with significantly lesser amounts in digalactosyldiglycerides (5.4%). This amounted to extremely high amounts of trienoic fatty acids in the mono- and digalactosyldiglycerides, 96.9% and 92.1%, respectively, with smaller amounts of linoleic (18:2), oleic (18:1) and palmitic (16:0). Allen et al. (1966) also reported linolenic acid (18:3) as the most abundant fatty acid in all other spinach lamellae lipid species except phosphatidyl inositol--which was, incidentally, the least abundant lipid--where palmitic acid was most abundant (33.5%). Phosphatidyl inositol was also found to have the largest concentration of hexadecatrienoic acid

(16:3) of all the identified phospholipids. This data was reaffirmed by Allen and Good (1971).

Two pathways of long chain fatty acid synthesis have been distinguished involving de novo synthesis and an elongation pathway (Mazliak, 1973). The de novo system was pioneered by Yang and Stumpf (1965) in the cytoplasmic supernatant of avocado mesocarp cells. Harwood and Stumpf (1972a) showed that palmitic acid is the product of the activity of the semipurified avocado fatty acid synthetase. The utilization of malonic acid of malonyl S-COA for fatty acid synthesis has been demonstrated in the chloroplasts isolated from lettuce (Brooks and Stumpf, 1966) and spinach (Mudd and McManus, 1964).

Givan and Stumpf (1971) and Kannangara and Stumpf (1971, 1972) described the regulation of fatty acid synthesis in chloroplasts. Since Weaire and Kekwick (1970) found that fatty acids could not be synthesized in either the cytoplasm or the mitochondria, fatty acid synthesis would most likely occur in the chloroplasts (Mazliak, 1973). Harwood and Stumpf (1972b) distinguished two types of fatty acid syntheses in avocado mesocarp, one associated with the chloroplast lamella and the other with the chloroplast stroma (Harwood et al., 1971).

Kolattukudy (1970) suggested elongation mechanisms for synthesizing long chain fatty acid. Experiments with barley seedlings (Hawke and Stumpf, 1965) and pea or safflower showed that 10% to 55% of the fatty acids synthesized from acetate were saturated fatty acids ranging from C20 to C28. Labeling patterns for this process were similar to that observed in microsomes (Kolattukudy and Buckner, 1972; Hawke and Stumpf, 1965). James (1962, 1963) reported that unsaturated fatty acids are synthesized from saturated fatty acids by desaturases

requiring oxygen and reduced pyridine nucleotide.

Skriver and Thompson (1976) found no significant effect of oxygen concentration on the degree of fatty acid unsaturation in Tetrahymena pyriformis even with a thirty-fold change in oxygen concentration. The apparent lack of correlation between oxygen concentration and the level of fatty acid unsaturation in T. pyriformis is in sharp contrast with yeast (Brown and Rose, 1969) and higher plants (Harris and James, 1969a and 1969b). Skriver and Thompson (1976) thought it remarkable that a complex series of reactions involved in the unsaturation of fatty acids could occur over a thirty fold range in oxygen concentration. Two explanations were noted. Either the existing molecules of desaturases are not limited by the oxygen concentration even at very low oxygen concentrations or the cell is able to synthesize more desaturase molecules as needed to maintain the proper ratio of saturated to unsaturated fatty acid. Rangi et al. (1975) found T. pyriformis has an increased capacity for utilizing oxygen under hypoxic conditions in apparent support of the first explanation.

Nagai and Bloch (1968) reported that a derivative of stearate was desaturated in spinach chloroplasts. Mazliak (1973) noted that no desaturases had yet been isolated from higher plants. Zilkey and Cavin in 1972 (see Mazliak, 1973) reported the presence of the enzymes necessary for oleic acid synthesis in proplastids of developing castor beans. Stearic acid was the precusor for oleic in this biosynthetic process (Rinne and Cavin, 1971).

Canvin (1965) found that polyunsaturated fatty acids were only slightly labeled when ^{14}C-acetate was used as a precursor. Further data, however, clearly indicated a progressive desaturation of oleic

acid to linoleic (18:2) to linolenic (18:3). Howling et al. (1972) cited evidence that only those monoenoic acids with double bonds in the ninth position could be converted into dienoic acids suggesting the presence of two desaturases differing in their ability to recognize substrates by the double bond position. The inhibition of linoleic acid synthesis by potassium cyanide without affecting oleic or linolenic acid synthesis suggested independent pathways for linoleic and linolenic acid in chloroplasts (Mazliak, 1973).

The non-random distribution of fatty acids among the different lipid species is accompanied by their non-random distribution between the one, two and three positions on the glycerol molecule.

Nearly all of the fatty acids produced are eventually incorporated into acyl lipids by fatty acid transferases which transport them from their site of synthesis to the site of lipid synthesis. Most of the accumulated evidence indicates that phosphatidic acid is the initial acceptor of acyl groups by the stepwise acylation of L-α-1-glycerophosphate with COA fatty esters.

The presence of dihydroxyacetone phosphate suggested that the acyl lipid may be derived either from the reaction of dihydroxacetone with ATP or glyerophosphate through glycerophosphate dehydrogenase activity.

Several groups of workers (Gurr and James, 1975; Hitchcock and Nichols, 1971; Mazliak, 1973) have published supportive evidence that point to phosphatic acid as the precursor of plant triglycerides. The three fatty acid residues of triglycerides are thought to be incorporated sequentially onto the glycerol rather than concomitantly. A different enzyme may be involved for each residue. The first two

residues are probably inserted by glyceryl-1-phosphoryl acyl transferases which control the acylation of the one and two positions while the third residue is probably inserted by a diglyceride acyl transferase. The evidence that different transacylases function most efficiently for specific fatty acids implies that the structure of specific lipids is not random but is controlled by the enzyme involved. There are, of course, different biosynthetic pathways for most lipid species.

MGDG and DGDG are the two major lipid constituents of chloroplast membranes (Ferrari and Benson, 1961; Allen et al., 1966; MacKender and Leech, 1974; and many others) and many attempts have been made to determine their biosynthetic pathways and functions. Studies on the incorporation of $^{14}CO_2$ into the lipids of Chlorella during photosynthesis (Ferrari and Benson, 1961) produced evidence that MGDG is the most rapidly labeled lipid followed by DGDG, which suggested to them that DGDG is formed by galactosylation of MGDG. From their experiments, Ferrari and Benson (1961) proposed a biosynthetic pathway for MGDG and DGDG involving UDP galactose. Neufeld and Hall (1964) tested this scheme with isolated spinach chloroplasts and reported that ^{14}C-UDP-galactose resulted in ^{14}C-labeled MGDG and DGDG. These results were confirmed by Ongun and Mudd (1968) and Mudd et al. (1969).

In 1970 Douce and Guillot-Solamon (see Mazliak, 1973) found that ^{14}C-labeled glycerophosphate is incorporated into MGDG in spinach chloroplasts or corn etioplasts provided UDP-galactose is present but remained in lyso- and phosphotidic acid and di- and triglycerides in its absence. No ^{14}C-labeled DGDG was found, however, suggesting that the first galactosylation producing MGDG is catalyzed by a different

enzyme than the second galactosylation forming DGDG. Ozone was found to be an inhibitor of UDP-galactose incorporation into MGDG (Mudd et al., 1971).

No definitive evidence had been produced from in vivo studies to confirm this proposed pathway or the pool of MGDG from which DGDG is formed. Williams et al. (1975) were probably the first to attempt an in vivo analysis of MGDG and DGDG synthesis. Their results seemed to support the formation of DGDG from galactosylation of MGDG via JDP-galactose. In addition, their results indicated that the galactose molecules of both galactolipids do not turn over rapidly but are fairly stable entitles supporting previous data by Tremolieres in 1970 (see Williams et al., 1975a) and Ferrari and Benson (1961).

Previous workers had shown that the synthetic mechanisms of MGDG and DGDG differ significantly. For example, Eccleshall and Hawke (1971) obtained significant synthesis of MGDG but little DGDG in spinach chloroplasts. Ongun and Mudd (1968) found that the enzymes that synthesize MGDG in spinach chloroplasts appear more tightly bound to the chloroplast membranes than the enzyme(s) responsible for DGDG synthesis. Mudd et al. (1969) reported a difference in the rate of synthesis of the two galactolipids at different pH's and Mudd et al. (1971) reported differences in the effects of inhibitors on the biosynthesis of the MGDG and DGDG in spinach chloroplasts supporting the conclusion that at least two enzymes exist. The data suggested that not only do the enzymes react differently to pH and various inhibitors but they seem to exist in different parts of the chloroplast. Data of Williams et al. (1975) clearly supported these conclusions indicating that DGDG is not synthesized via a two enzyme system which synthesizes

DGDG from MGDG in rapid succession. Rather DGDG synthesis seemed to involve a separate process (or processes) involving galactosylation of two (or more) pools of MGDG resulting in a relatively slow production of DGDG (Williams et al., 1975).

Data accumulated by Williams et al. (1976) suggested that MGDG was synthesized by galactosylation of a diglyceride containing high concentrations of unsaturated fatty acids--especially linolenic acid. This data agreed with Mudd et al. (1969) who showed a specificity of the galactosylation enzyme for diglycerides containing unsaturated fatty acids. On the contrary, Eccleshall and Hawke (1971) found that synthetic diglycerides which differed in fatty acid composition gave the same rate of galactose incorporation from UDP-galactose as diglycerides with highly unsaturated fatty acids. Although the origin of the more saturated fatty acids found in MGDG are unclear, the labeling kinetics of palmitic acid in PG suggested that PG may be a source of palmitic acid for MGDG (Williams et al., 1976). Results by Williams et al. (1976) also indicated that DGDG and MGDG are not synthesized by galactosylation of diglycerides from a common pool.

The ratio of thylakoid membrane to envelope membrane increases dramatically during chloroplast development. One could assume from this observation that any lipid species important to photosynthesis might also appear and/or increase concentration during that time. Leese and Leech (1976) found a six-fold increase in galactolipids during chloroplast development. Galactolipids increased from 66% to 90% of the total lipid with MGDG increasing faster than DGDG with a molar ratio of 1:1 in younger plastid to 2:1 in older plastids.

Jost et al. (1973) suggested that membrane lipids are differentiated beyond the inner and outer layers of the lipid bilayer into the so-called boundary lipid which are fixed into position surrounding the outside hydrophobic regions of the membrane proteins and the more fluid lipids of the matrix of the bilayer. The neutral galactolipids are the most likely candidates for the fluid bilayer of chloroplast membranes based on their uncharged polar head groups (Anderson, 1975b) and the high level of unsaturation of their fatty acids.

Hubbel and McConnell (1971) and Eletr and Keith (1972) suggested that the greater the degree of unsaturation of membrane lipid fatty acids and the further the double bonds are located from the point of esterfication, the greater the degree of membrane fluidity. The reported high levels of unsaturation of galactolipids and the localization of double bonds near the tail ends of the fatty acids would make the membrane bilayer fluid in the matrix region if they were indeed the predominant matrix lipid (Anderson, 1975b).

Anderson (1975b) further suggested that the available evidence supports the involvement of galactolipids as the predominant species of the fluid matrix of chloroplast membranes. Treatment of chloroplasts with crude galactolipase prior to freeze-fracture destroyed the usual freeze-fracture membrane pattern. Billecocq (1974) showed that antibodies attached to membrane galactolipids were distributed not homogeneously but in discreet patches on the external thylakoid surface. However, this experiment did not show whether the galactolipid formed the lipid matrix or the lipoprotein areas. Treatment of the membrane with galactolipase liberated substantial amounts of

galactolipids from the subchloroplast fractions without inhibiting electron transport if bovine serum albumin (BSA) was present to tie up the liberated fatty acids (Anderson et al., 1974). This indicated that galactolipids are not an integral part of photosystems I and II. Anderson (1975b) even suggested the possibility that galactolipids may be asymmetrically located across the membrane lipid bilayer since the mass of their head groups differ as do their pattern of acylation. Anderson et al. (1974) and Helmsing (1969) found that galactolipase acts on both MGDG and DGDG. In 1972, Radunz used a mono-specific antiserum for MGDG and showed that some MGDG was present at the external thylakoid surface. In a 1973 study of the effects of pH and ionic strength on spinach chloroplast membranes, Heise and Jacobi found more MGDG on the external thylakoid surface than DGDG (see Anderson, 1975b).

Shaw et al. (1976) studied the effects of lipase isolated from the primary leaves of Phaseolus vulgaris on isolated spinach chloroplasts. Chloroplasts treated with lipase in the absence of BSA lost 91% of their MGDG, 83% of the DGDG, all of their PC, but none of the SL or PG. Electron microscopy indicated that lipase treated chloroplasts were swollen and had lost their characteristic membrane stacking. Chlorophyll, carotenoids, and coupling factor 1 (CF_1) remained bound to the treated membranes. Nearly total inhibition of all photochemical activities occurred when the chloroplasts were treated with lipase in the absence of BSA, probably due primarily to the release of fatty acids (especially linolenic acid) from the membranes (Krogmann and Jagendorf, 1959; Cohen et al., 1969; and Siegenthaler, 1973). Shaw et al. (1976) suggested that even with BSA present, the inhibition of oxygen evolution and photophosphorylation by fatty acids could be the result of the

binding of the lipase-released fatty acids to the membrane due to the competition between BSA and the membrane to bind the liberated fatty acids. This theory was supported by reduced sensitivity of photophosphorylation to lipase when the enzyme was added in small aliquots over an extended period.

In the presence of BSA, which retained 30% of the MGDG and 50% of the DGDG and PC, Shaw et al. (1976) found that electron flow through photosystems I and II was apparently unaffected by lipase treatment while oxygen evolution and photophosphorylation were partially inhibited. Although inconclusive, this data suggested that galactolipids have little if any effect on photophosphorylation but may play some role in electron flow and oxygen evolution. It was difficult to assess the role(s) of galactolipids in these processes due to the irreversible inhibitory effects of fatty acids. The possible major role of galatolipids in the construction of the photosynthetic apparatus still has not been elucidated (Shaw et al., 1976).

Effects of Supra Optimal Temperature on Chloroplast Lipids

Heilbrunn (1924) and Belehradek (1931) reported that the physical state of membrane lipids, which is a function of temperature, plays a vital role in determining the maximum and minimum temperatures at which an organism can survive. Gaughran (1947) postulated that cells cannot grow at temperatures below that temperature at which their membrane lipids solidify. Brock (1967) and Ingraham (1962) suggested that thermal death is a first order process which implies that a single event within the cell causes cell death. Since there is a complex

network of enzymes within the cell, it seemed unlikely that the loss of activity of a single enzyme would result in immediate cell death. It appeared more likely to them that cell death occurred as a result of damage, disruption, or destruction of some essential cellular macrostructure, most likely the membrane.

Since membranes contain large amounts of polar lipids (MGDG, DGDG, SL and PL) (Ongun and Mudd, 1968; Rothfield and Finkelstein, 1968, and many others), variations in these membrane components might reflect a change in the thermostability of cells (Kleinschmidt and McMahon, 1970b). Working with the thermophilic eucaryote, Cyanidium caldarium, which grows at temperatures ranging from less than 20°C to 56°C, Kleinschmidt and McMahon (1970) reported significantly reduced amounts of total lipids and fatty acids at higher temperatures with little change in lipid distribution at the two growth temperatures. Results also indicated higher ratios of saturated to unsaturated fatty acids at higher temperatures agreeing with earlier studies by Farkas and Herodek (1964), Gaughran (1947), Holton et al. (1964), and Marr and Ingram (1962).

Kleinschmidt and McMahon (1970b) reported higher MGDG, DGDG, and SL in cells grown at 20°C than in those grown at 55°C. Since MGDG and DGDG are primarily associated with chloroplast membranes, their data was felt to point toward the photosynthetic apparatus as the critical factor in thermal damage in photosynthetic plants. This idea was supported by yellowing of cells grown at 55°C.

Fatty acid analysis of the galactolipids and SL of Cyanidium indicated significantly increased ratios of saturated to unsaturated fatty acids at higher temperatures. Major changes involved significant

increases in palmitic acid (16:0) at 55°C while linolenic acid (18:3) dropped to zero. Kleinschmidt and McMahon (1970b) suggested that the enzyme(s) converting linoleic acid (18:2) to linolenic acid (18:3) may be denatured or otherwise inactivated at higher temperatures. They then reasoned that if that were true, would it not logically follow that the same enzyme might also be involved in the conversion of oleic to linoleic and would also eliminate linoleic from the cell--which did not occur. Kleinschmidt and McMahon (1970b) suggested a control mechanism within the cell regulating the degree of unsaturation of membrane lipids. However, if this were true, one would still expect to find trace amounts of linolenic acid, which their data did not confirm. The question remained unanswered.

Although the exact role of lipids in cell membranes is obscure, Belehradek (1931, 1935), Brock (1967), Gaughran (1947), and others cited evidence implying that lipids are involved in maintaining the integrity of cell membranes at elevated temperatures. This theory was tested by Kleinschmidt and McMahon (1970b) who compared the effects of increasing temperature on the membrane integrity of Cyanidium cells grown at 20°C and 55°C. At the point of thermal disruption of cell membranes, electrolytes leaked out causing an increase in conductance of the cell suspension. The cells grown at 20°C were found to be more thermal sensitive than cells grown at 55°C showing loss of membrane integrity at around 10°C to 15°C below those grown at 55°C. This suggested a correlation between the degree of saturation of lipid fatty acids and membrane thermostability.

Contrary to previous data (Roots, 1968; Okuyama, 1969) showing no alteration in the proportions of membrane lipids with temperature,

Fukushima et al. (1976) found significant phospholipid changes in Tetrahymena as a function of temperature. Results showed a decrease in phosphonolipid concentration and a corresponding increase in phosphatidylethanolamine concentration as growth temperature increased. Lower temperatures were marked by increases in linoleic and linolenic acids and decreased palmitic acid.

The easily detectable temperature-induced changes in fatty acid patterns led Fukishima et al. (1976) to study the biochemical factors regulating temperature adaptation. C^{14}--palmitic acid--added as a tracer to cells grown at 39.5°C--was rapidly (82% in 5 minutes) incorporated into the membrane phospholipids. However, they reported over a 25-hour period, the migration of radioactivity into more unsaturated fatty acids, reaching 42% after 24 hours. This same general pattern was detected in cells grown at 15°C except incorporation was slower. This suggested a redistribution of radioactivity among all of the different species of membrane fatty acids. Thus newly synthesized fatty acid appeared to quickly become metabolically indistinguishable from pre-existing fatty acids as far as desaturase activity is concerned.

The most striking discovery of the work Fukushima et al. (1976) working with thermotolerant strain of Tetrahymena pyriformis was the unexpected 10% redistribution of phospholipids at changing temperatures. Nozawa et al. (1974) and Wunderlich and Ronai (1975) detected similar trends with much smaller levels of redistribution of phospholipids ranging from 1.8% to 3.2%. Thompson and Zalik (1973) detected large increases in linolenic acid and a decline in phosphatidyl choline in rye seedling following an eight-week vernalization at 4°C. Khuller

and Goldfine (1974) detected an increase in the concentration of phosphatidyl ethanolamine and N-phosphatidyl-methyetnanolamine from 35% of the total phospholipid in Clostridium butyricum grown at 25°C to 42% of the total phospholipid when grown at 37°C. The increase was primarily at the expense of phosphatidylglycerol.

Michaelson et al. (1974) and Jacobson and Papahadjopoulos (1975), using artificial membranes, cited evidence suggesting that changes in the polar head groups of membrane phospholipids can affect membrane fluidity. This led Fukushima et al. (1976) to conclude that the changes they observed in membrane phosopholipid composition of Tetrahymena strain NT-1 grown at high and low temperature represented an important adaptive process. The extent of the phospholipid change depended on the membrane involved.

Skriver and Thompson (1976) reported a lack of correlation between fatty acid desaturation and growth rate. They also reported evidence indicating that the cell is capable of synthesizing more desaturase molecules as needed to maintain the optimum membrane fluidity at that temperature. Fulco (1974) found that decreased temperature not oxygen concentrations triggered fatty acid desaturase synthesis in Bacillus megatorium. This conclusion was supported by evidence cited by Skriver and Thompson (1976) indicating no significant effects of oxygen concentration on the degree of fatty acid unsaturation over a thirty-fold change in oxygen concentration. This enzyme was found to be very temperature sensitive decreasing markedly with increasing growth temperature.

Working with Atriplex lentiformis (plants collected from a coastal habitat near Ventura, California, and a desert habitat near

Death Valley) Pearcy (1978) found a consistent correlation between growth temperature and the fatty acid composition of whole leaf lipids but temperature had little effect on the relative proportions of galactolipids and sulfolipids. High growth temperatures were found to consistently induce increased amounts of saturated fatty acids in whole leaf lipids. One qualitative difference in MGDG and DGDG reported was the presence of hexadecatrienoic acid (16:3) in the plants grown at $23^{o}C/18^{o}C$ day/night temperatures and its apparent absence in plants grown at $43^{o}C/30^{o}C$ day/night temperatures, especially in MGDG. Significant decreases in linoleic (18:3) in the order of 10% to 20% occurred in other leaf lipids with concomitant increases in more highly saturated fatty acids. Growth temperature had no apparent effect on the 18:3 concentration in MGDG.

The moderate increases in lipid saturation observed by Pearcy (1978) correlated with the increased thermal stability of PSII with increased temperature reported by Pearcy et al. (1977). The greater lipid saturation observed here might be expected to increase thermal stability of membranes due to the higher melting temperatures. The high proportion of 18:3 even in plants grown at high temperatures would appear to rule them out as important factors in the thermostability of membranes (Pearcy, 1978). Furthermore, Pearcy (1978) stated that if lipids are important in conferring thermostability to photosynthesis, the mechanism involved is more complex than any presently proposed. Pearcy (1978) noted that the theory postulated by Anderson (1975) identifying the galactolipids as components of the membrane fluid bilayer and SL and PG as protein boundary lipids could be significant. SL and PG are much more saturated than the galactolipids and showed

relatively greater changes in 18:3 in response to growth temperature (Pearcy, 1978). This may support the proposed involvement of these lipids in conferring thermal stability of chloroplast membranes as proposed by Bjorkman et al. (1976).

Effect of Temperature on Fluidity and Phase Changes

Biological membranes are believed to exist as liquid crystalline lipid bilayer in which proteins are imbedded (Singer and Nicholson, 1972). The degree of fluidity of these biological membranes is related to lipid composition (Mitranic et al., 1976) and must be maintained within rather narrow limits to ensure proper membrane function (Kimelberg and Papahadjopoulous, 1974).

Environmental factors can markedly affect membrane fluidity, especially in poikilothermic organisms where cell temperature fluctuations induce profound changes in membrane physical properties (James and Branton, 1973). Many cells have the remarkable capacity to alter their membrane lipid composition in responses to environmental temperature fluctuations and to maintain the level of fluidity necessary for membrane function (Roots, 1968).

The idea that the maximum temperature an organism can survive is determined by its membrane stability has been proposed by numerous investigators. Heilbrunn (1924) and Belehradek (1931) postulated that organisms adapt to changes in temperature by altering their plasma lipid composition and that heat resistance was related to the melting temperature of the lipids. It is interesting to note that at the time

of their proposals, the presence of lipids in membranes was not known.

Johnston and Roots (1964) suggested that cells must maintain membrane lipids near the critical point of phase transition to achieve the necessary level of fluidity. They further suggested that varying the degree of fatty acid unsaturation could provide an effective means of preserving this state, an idea incorporated into the theory of membrane structure by Singer in 1971.

The importance of lipid phase transitions to the thermal stability of membranes of thermophilic micro-organisms has been supported by the work of Desiervo (1969) and Daron (1970) who found an inverse relationship between the degree of unsaturation and growth temperature. Data presented by Esser and Souza (1974) strongly indicated that preservation of the physical state of the lipids in the plasma membrane of Bacillus steareothermophilus is a major reason for temperature induced changes in lipid composition. Earlier work by Chan et al. (1973) was interpreted to indicate that membranes of cells grown at higher temperatures are more rigid than those grown at lower temperature. Esser and Souza (1974) agreed with this conclusion only if the comparison is made at temperatures below the growth temperature.

What advantage to the cell is a mechanism to alter the composition of its lipids in response to temperature? Overath et al. (1971) suggested that there exists an upper limit to phase fluidity that is compatible to growth. This is reminiscent of the older theories of Heilbrunn (1924) and Belehradek (1931) which stated that the maximum growth temperature an organism can survive is determined by the melting points of its lipids. Esser and Souza (1974) proposed that the temperature

limit for thermophilic bacteria growth may be determined by the phase conditions of the total lipid mixture in the membrane. Several earlier investigators suggested on theroretical grounds that lipids in biological membranes must exist in the liquid-crystalline state in order for the membrane to function properly (Chapman, 1967). Experimental data seems to support their proposal. Wild type cells of Bacillus stearothermophilus responded to a shift in growth temperature from 42°C to 65°C by an increase, from 42% to 69% in its membrane fatty acids with melting points above 55°C which represents a shift to increased levels of saturation. A mutant temperature sensitive strain of Bacillus stearothermophilus was severely limited in its ability to make changes in its membrane lipids above 52°C. The boundary temperatures for the temperature sensitive strain based on the boundaries for phase separation of the membrane lipids are 52°C and 58°C when grown at 52°C. Thus, if their theory is correct, the growth limit of the mutant grown at 52°C should be 58°C. Data confirmed this theory (Souza et al., 1974).

Overath et al. (1971), Linden et al. (1973) and Tsukagoshi and Fox (1973) presented evidence that the assembly of membranes of E. coli does not proceed correctly when the lipids are in an all "frozen" state which occurs below the temperature where phase separation begins. Overath et al. (1971) postulated that the shift in phase to the fluid state must occur for proper insertion of lipids and proteins into the membranes. The fluid state assures a rapid, completely random insertion of lipids into the membranes (Esser and Souza, 1974). Contradictory to this idea of random insertion of the lipids into the membrane is the demonstrated requirement for specific lipid halos for certain enzymes (Rothfield and Romeo, 1971).

McElhaney (1974) found that the transition temperature of the lipid components of Acholeplasma laidlawii membranes depend on the fatty acid composition of these membranes. About 90% of their membrane lipids were found to participate in this phase transition. Since the melting of the hydrocarbon chain is apparently a cooperative phenomenon affected by hydrophobic interactions with proteins or sterols, it was concluded that the majority of the lipid hydrocarbon chains in this organism interact hydrophobically with the hydrocarbon chains of adjacent lipid molecules and not with membrane proteins (see McElhaney, 1974).

Steim et al. (1969) presented evidence that cell swelling and lysis that occurred when A. laidlawii was grown in the presence of long chain saturated fatty acids may have resulted from a crystallization of hydrocarbon chains of the membrane lipids. These experiments were limited in scope and left certain questions unanswered, especially the relationship between membrane fluidity and membrane function.

McElhaney and Tourtellotte (1969) found that by manipulating the fatty acid content of the growth media they could alter the fatty acid composition of the organisms, making it possible for them to study cell growth at temperatures where either the gel or liquid-crystalline phase exist exclusively and within the phase transition range where various proportions of both phases exist simultaneously.

The effects of altering growth temperature on the fatty acid composition of the total membrane lipids were investigated. The results, when no exogenous fatty acids are supplied, indicated a slight increase in chain length of membrane lipids with increased growth temperature. Significant increases in fatty acids did not occur with temperature when oleic acid was added to the growth medium indicating that the

enzyme systems responsible for fatty acid biosynthesis in A. laidlawii are sensitive to environmental temperature only in the absence of exogenous fatty acids. Those enzymes involved in the uptake and incorporation of exogenous fatty acids were not significantly affected by changes in environmental temperatures (McElhaney, 1974).

Since the magnitude of the temperature differential at any temperature within the phase transition range is proportional to the amount of lipid undergoing the phase transition at that temperature, the progress of the phase transition can be monitored by thermal analysis.

McElhaney (1974) suggested that the relationship between the optimum and maximum growth temperatures and the physical state of the membrane lipids is indirect since he could not identify any definite and constant relationship between growth and the membrane lipid transitions. This led McElhaney (1974) to conclude that the maximum growth temperature is related to one or more cellular proteins. He did, however, concede that membrane lipids do have some effect on growth since cells supplemented with fatty acids exhibited shifts in optimum and maximum growth temperatures of the cells accordingly. This was interpreted as supportive evidence of upper and lower limits on membrane lipid fluidity compatible for growth.

All membrane lipids do not exist in the same state at any temperature but the cells are able to function as long as enough of the lipids maintain the required level of fluidity. The temperature dependence of growth is determined by the temperature dependence of one or more essential metabolic reactions occurring within that portion of the membrane. Cells grown above the phase transition midpoint temperature have an excess of fluidity.

Several recent studies support the idea that the liquid-crystalline state is necessary to support growth, membrane transport (Overath et al., 1970), and the activity of membrane associated enzymes (Kimelberg and Papahadjopoulos, 1972).

The fatty acid chains of membrane lipid seem to undergo phase transitions at specific sites. Arrhenius plots of activity of several membrane bound enzymes of various organisms exhibit breaks or kinks which have been attributed to phase changes in the lipid portions of the membrane. These phase changes are thought to induce conformational changes in the enzyme--a conclusion which had only limited experimental support until DeKruyff et al. (1973) presented supportive evidence that the breaks in the Arrhenius plots are associated with membrane lipid transition in A. laidlawii. These phase transitions were apparently induced by a gradual melting of various membrane lipids. Those with the lowest transition temperature melted first followed in order by those with progressively higher transition temperatures. The temperature at which the breaks occurred in the Arrhenius plots apparently correspond to the transition temperature of that species of membrane lipid with the lowest transition temperature.

DeKruyff et al. (1973) suggested that the apparent preferential association of membrane ATPase with specific membrane lipids might best be explained as follows:

At temperatures where the membrane is in the required liquid-crystalline condition where proper fluidity is maintained, the enzyme may be randomly associated with all the membrane lipids. Under these conditions, there is a rapid exchange between the liquid-crystalline species associated with the enzyme and the liquid-crystalline lipid

species present in the membrane. A key assumption here is that there is a preferential association between the enzyme and lipids in the liquid-crystalline state over association with those membrane lipids in the gel state. Under conditions of changing temperature, the enzyme exchanges that lipid in the gel state for one in the liquid-crystalline state. This exchange continues until the lower end of the phase transition is reached. When the lowest transition temperature is reached where all available membrane lipids are in the gel state, the enzyme undergoes configurational changes causing increased activation energy of the enzyme as suggested by the "kink" in the Arrhenius plot.

Phillips (1972) suggested another formulation of this theory based on the preferential association of ATPase with only those lipids in the liquid-crystalline state due to the tendency for lipids in the gel state to separate in the membrane, a theory supported experimentally by the work of Verkley et al. (1972). They demonstrated the presence of particles in A. laidlawii membranes using freeze etch electron microscopy which became aggregated at temperatures corresponding to the phase transition temperature. Verkley et al. (1972) identified these particles as proteins which are excluded from the lipid portion in the gel state.

It is interesting to note at this point that the addition of cholesterol induces temperature shifts in phase transitions, apparently decreasing the temperature of the Arrhenius plot kink and the starting temperature of the lipid phase transition. If one assumes that cholesterol interacts randomly with all molecular species of lipids present in the membrane, this would result in a general broadening of the phase transition of uncomplexed lipids pushing the lower phase transition temperature downward and the upper transition temperature upward.

However, DeKruyff et al. (1973) could not demonstrate this upper temperature phase shift. In fact, some data showed a slight downward shift in transition temperatures (DeKruyff et al., 1972).

Increases in ratio of saturated fatty acids to unsaturated fatty acids and shorter fatty acid length with increasing temperature apparently increase the temperature of phase change which occurs following the melting of hydrocarbon chains in the interior of the lipid bilayer. These chemical alterations of the membrane fatty acids minimize the changes in fluidity and phase transition of the membrane lipids.

E. coli has been shown to be able to maintain the proper fluidity level over its entire growth range. This phenomenon is termed homeoviscous adaptation (Sinensky, 1974). Since the permeability properties of cellular membranes (McElhaney et al., 1973), the activity of certain membrane bound enzymes (Kimelberg and Papahadjopoulos, 1972) and the transport systems (Overath et al., 1970) are markedly dependent on the fluidity and physical state of the membrane lipids, it appears that homeoviscous adaptations represent an important mechanism for ensuring optimum growth over a wide range of temperatures. McElhaney and Souza (1976) demonstrated (using the growth rates of wild and temperature sensitive Bacillus) the inability of the mutant to grow at the optimum and maximum temperatures of the wild type Bacillus. Analysis of membrane lipid fatty acids of the wild type and mutant Bacillus grown at 42°C showed the expected increase ratio in saturated fatty acids (especially palmitic) to unsaturated fatty acids ratios. When grown at 52°C, the mutant did not make the expected increases in saturated fatty acid/unsaturated fatty acids.

Mitranic et al. (1976) cited evidence from freeze fractured

sections examined by electron microscopy suggesting that supra optimal temperatures induced alterations of Golgi membrane structure. Data indicated that the membrane or plane of cleavage through the membrane varies with temperature possibly due to changes in the lipid-lipid or lipid-protein interaction within the membrane. Barnett et al. (1974) noted that the appearance of freeze-fractured membranes correlates with the fluidity of membrane lipids suggesting that the altered behavior of Golgi membranes at different temperatures may reflect the physical state of the membrane lipids.

Both galactosyl transferase and sialyl transferase are Golgi membrane bound enzymes. Arrhenius plots showed kinks in the sialyl transferase at 28°C and thermal denaturation above 43°C while no kinks were observed in the Arrhenius plot of the galactosyl transferase up to 45°C where an abrupt slope change with rapid loss in enzyme activity was again interpreted as thermal denaturation.

This data suggested that the two enzymes have different lipid environments. Since the Arrhenius plot for galactosyl transferase showed no phase transition, as represented by a straight line, it was thought to possess a more liquid environment than sialyltransferase. The importance of the fluidity of the lipid environment may be to maintain the correct conformation of the membrane bound enzyme (Kimelberg and Papahadjonoulos, 1974). In the case of galactosyltransferase, the lipid environment of the enzyme appears to provide access of lipid intermediates to the enzymes in the transferase reactions. Access to the appropriate transferase was thought to be facilitated by a liquid-crystalline lipid environment around the enzyme.

Data collected by Kimelberg and Papahadjopoulos (1972) and

Grisham and Barnett (1973) suggested that ($Na^+ + K^+$) stimulated ATPase activity is strongly affected by the thermotropic phospholipid phase transitions while the Mg^{+2} activated ATPase seemed to be unaffected by these phase transitions.

It is well known that the phase transition temperatures of a lipid varies with the carbon length of the fatty acid and the number of double bonds. This explains why the same lipid species may have different temperatures for phase transitions. Kimelberg and Papahadjopoulos (1974) presented evidence which they felt implied that Arrhenius plots suggested a lower limit transition from solid to a mixed liquid-crystalline state and an upper transition to a completely fluid phase, where the enzyme is in a homogeneous lipid fluid environment. The temperature for these "kinks" would be determined by the composition of the lipid portion of the membrane. Data also suggested that both Na^+ permeability and ATPase activity are more sensitive indicators for the onset of the fluid state than differential scanning colorimetry.

The heterogeneous lipid composition of biological membranes makes a separation of the membrane lipids into different domains reasonable either on the basis of differential binding to proteins or their widely different melting points (Phillips et al., 1972). These different micro-environments within biological membranes were suggested by Esfahani et al. (1971) who found the kinks in their Arrhenius plots occurred several degrees below the phase transition temperature of E. coli as determined by x-ray diffraction. Similar conclusions were reached independently by Mavis and Vagelos (1972) and Lenaz et al. (1972).

The presence of the divalent cation Mg^{+2} at concentrations equal to those in the reaction mixture for ATPase activity shifted the transition temperature from 40°C to 50°C. This effect can be reversed by equimolar amounts of ATP (Kimelberg and Papahadjopolous, 1974).

No matter how the Arrhenius plots are interpreted, the activity of membrane bound enzymes appears to have an overall dependence on lipid fluidity (Kimelberg and Papahadjopolous, 1974) as previously established by Wilson et al. (1970), Overath et al. (1970), Esfahani et al. (1971), and Mavis and Vigelos (1972).

The inhibitory effects of cholesterol on ($Na^+ + K^+$) activated ATPase activity was shown by Papahadjopoulos et al. (1973) to be due to its apparent ability to inhibit the ability of various proteins to expand to the area of the phospholipid monolayer and to increase the permeability of vesicles. This could mean that cholesterol inhibits ATPase activity by inhibiting the ability of the protein to enter the lipid bilayer. A second possibility could be due to decreased molecular motion or conformational freedom for the enzymes still partially embedded within the lipid bilayer by lowering the temperature below the phase transition temperature on the addition of cholesterol (Kimelberg and Papahadjopoulos, 1974).

Kimelberg and Papahadjopoulos (1974) found ATPase activity to be only partially inhibited by cholesterol in the presence of unsaturated fatty acids while its activity in the presence of saturated fatty acids is completely inhibited by cholesterol, generally agreeing with previous studies by Chapman et al. (1969) and Demel et al. (1972).

The influence of fatty acyl chain fluidity on enzyme function discussed by Kimelberg and Papahadjopoulos (1974) indicated an

important but subtle involvement of lipids in cell function for which they coined the word "viscotropic."

The fluidity of membrane lipids can be controlled by cholesterol, fatty acyl chain length, unsaturation or saturation. This fluidity could have an important controlling effect on the activity of membrane bound enzymes. By controlling the activity of certain membrane bound enzymes like ATPase, lipid fluidity could have a controlling effect on general cell metabolism.

It is possible that changes in membrane lipid fluidity could be intimately involved in physiological control plus a possible involvement in pathological situations. Cholesterol could be considered a stabilizing force for membrane integrity. Increased concentrations of cholesterol in membranes due to a pathological condition could interfere with the activity of certain membrane bound enzymes such as $(Na^+ + K^+)$ activated ATPase with obvious detrimental effects on cell metabolism, especially in animals.

Murato et al. (1975) reported that until that time, there had been no good demonstration of a meaningful relationship between the phase transition of membrane lipids and photosynthetic activities. The high linolenic acid content of the major chloroplast galactolipids suggested that the phase transition would occur far below room temperature (Murato et al., 1975) in higher plants. Shipley et al. (1973) showed that the phase transition of MGDG occurs at $-30^{o}C$ and at $-50^{o}C$ in DGDG extracted from Pelargonium. Holton et al. (1964), Hirayama (1967) and Nichols et al. (1965) showed that in the blue-green algae Anacystis nidulans the major fatty acids were palmitic and hexadecanoic acid (85% - 90%) and no linolenic acid and that the fatty acid

composition varied depending on the growth temperature. The higher the temperature, the higher the content of saturated fatty acids (Holton et al., 1964).

Murata et al. (1975) established that membrane phase transitions induced characteristic changes in photosynthetic parameters of Anacystis nidulans as reflected by the appearance of maximum yield of chlorophyll a fluorescence, a change in the activation energy for the electron transport reaction, and configurational changes in the thylakoid membranes. Data indicated that organisms grown at 28°C showed phase transition temperatures between 10°C and 13°C while organism grown at 38°C showed phase transition temperatures between 18°C and 24°C. Murato et al. (1975) also observed difference patterns of photosynthetic parameters in plants grown at 28°C and 38°C, and concluded that they were responding to phase transitions in the membrane lipids. Note that these phase transitions differed with growth temperature and are apparently dependent upon growth temperature.

Murata et al. (1975) identified the site of electron transport influenced by the transition of the lipid phase of the thylakoid membrane as the oxidation of plastoquinone. The appearance of a change in the rate of P_{700} reduction corresponding to a transition in the phase of membrane lipids led to the conclusion that this part of the electron transport system involving plastoquinone is influenced by the phase transition of lipids. The fact that plastoquinone is the most lipophilic component of the electron transport system suggests that plastoquinone is most likely to be influenced by the fluidity of membrane lipids. This conclusion is supported by data concerning oxidative electron transport in mitochondria (Raison et al., 1971) and in

bacterial membranes (Esfahani et al., 1972) which indicated that the oxidation-reduction reactions show a transition in activation energy related to the transition phase of the membrane lipids. Murata et al. (1975) also felt that plastoquinone is the most likely rate limiting site between photosystems I and II in lettuce chloroplasts and the probable site of heat sensitive lipid phase transition. Murato et al. (1975) observed that the state I to state II shift of light distribution between the two pigment systems is highly dependent on the fluidity of membrane lipids almost disappearing when the membrane lipids are in the mixed solid-liquid-crystalline state.

Comparison of changes in chlorophyll a fluorescence of lettuce chloroplasts grown at 25^{o} and treated at $6^{o}C$ and $38^{o}C$ in the presence of the divalent cation Mg^{+2} showed that the higher temperature treatment induced a significant increase in chlorophyll a fluorescence suggesting that fluorescence change is very heat sensitive. The divalent cations probably cause a configurational change in the thylakoid membranes which is expressed by a change in the efficiency of transfer of excitation energy between the two pigment systems (Murata, 1971).

The lack of "kinks" in the Arrhenius plot suggests that the fluidity of membrane lipids in spinach chloroplasts is independent of growth temperature (Murata et al., 1975).

Murata and Fork (1975) found that the fluorescent yield of chlorophyll in some algae shows characteristics relating this phenomenon to phase changes of membrane lipids. These findings were consistent with those of Colbow (1973) which showed that chlorophyll a in a phospholipid model membrane acts as a fluorescent probe for the detection of temperature induced phase changes of membrane lipids. The fluorescent yield

of chlorophyll a is drastically decreased as the temperature passes through the phase transition temperature moving from high to low.

The different transition points of Anacystus grown at different temperatures were thought to correspond to the change in phase from liquid-crystalline to mixed solid-liquid-crystalline. Thus the transition temperature here corresponded to the beginning of solidification of the membrane lipids. The temperature of the maximum fluorescent yield was close to this phase transition temperature especially when the temperature was increased.

Holton et al. (1964) found a correlation between growth temperature of Anacystis and the degree of saturation of membrane lipids. Saturation decreased with decreased growth temperature. The phase transition temperature was shown to vary with growth temperature and fatty acid composition of the thylakoid membranes. Increasing unsaturation of fatty acids corresponded to lower transition temperature while increasing saturation of fatty acids corresponded to higher transition temperatures.

The study of artificial fluorescent probes in model membranes suggests that they are partitioned between the hydrophobic region of the membrane and the aqueous phase. These probes fluoresce when they are bound to the membrane (Overath and Trauble, 1973). The hydrophobicity is higher when the membrane is in the liquid crystalline state than when in the solid state. Murata and Fork (1975) suggested that when the temperature passes through the phase transition from high (liquid crystalline) to low (solid) the number of probe molecules decreases with a concomitant abrupt decrease in chlorophyll a chlorophyll a fluorescence. Chlorophyll consists of a tetraporphyrin ring with

hydrophobic and hydrophilic regions and a long chain alcohol (phytol) which is hydrophobic. Thermodynamically, the most stable condition of the chlorophyll a molecules in the lipid bilayer occurs when the phytol chain is associated with the hydrocarbon region of the membrane lipids and the porphyrin ring is located at the membrane surface. The chlorophyll molecules are always bound to the thylakoid membrane because of the strong interaction between the phytol and the hydrocarbon region regardless of the change in hydrophobicity of the membrane produced by a change in the physical phase of the membrane lipids. More of the porphyrin ring is dissolved in the hydrocarbon portion of the membrane while in the liquid crystalline state than in the solid state. It seemed likely to Murata and Fork (1975) that only that chlorophyll a bound to the thylakoid membrane lipid is directly influenced by the physical state of those same lipids while that chlorophyll a bound to the membrane proteins remains unaffected. However, Murata and Fork (1975) added that the transfer of excitation energy between the lipid bound chlorophyll a and protein bound chlorophyll a molecules must be very fast and efficient. Therefore, chlorophyll a fluorescence from both kinds should respond to lipid transition in similar ways.

Using fluorescence of chlorophyll a in vivo as a means of detecting changes in the physical state of thylakoid membrane lipids, Fork and Murata (1977) studied the effects of temperature on the thermophilic blue-green alga Senechococcus lividus. Results indicated significant alterations in the rates of photosynthetic electron transport above and below the transition temperature. Fork and Murata (1977) observed the temperature dependence for transient reduction of cytochrome f during exposure to light. The observed change in the rate

of cytochrome f reduction following heat treatment apparently corresponds to a phase transition in the membrane lipids, suggested that part of the photosynthetic electron transport involving the lipophilic plastoquinone molecules is significantly affected by changes in the phase of the thylakoid membrane lipids. This study indicated that the maintenance of the proper fluidity of the thylakoid membrane lipids is essential for the necessary electron flow in photosynthesis. It also supported previous reports of the ability of some organisms to adapt to changed environmental conditions.

Effects of Supra Optimal Temperature on Chloroplast Function

A close correlation exists between the morphological state and the activity of energy transforming cellular organelles such as mitochondria and chloroplasts. Heating a suspension at $40^{o}C$ causes isolated chloroplasts to swell. This swelling can be reversed by adding ATP and Mg^{+2} to the suspension (Molotkovsky and Zheskoya, 1965). The effects of ATP were intensified by the addition of bovine serum albumen (BSA). The effects of ATP were temporary; the chloroplasts swelled as the ATP was used up. The addition of ATP again caused contraction of the chloroplasts followed again by swelling. The heat injured chloroplasts were restored to their initial morphological state when an excess of ATP was added. As the ATP was consumed, swelling reoccurred.

Molotkovsky and Zheskoya (1965) compared the contraction of swollen chloroplasts to a similar process in mitochondria observed by Lehninger (1962). This comparison was justified by the isolation of an

actinomyosin-like protein from chloroplasts (Ohnishi, 1964) similar to that found in mitochondria. Heat induced swelling of the chloroplast as ATP was utilized. The addition of ATP and Mg^{+2} replenished the depleted ATP affecting the mechanochemical processes necessary for contraction of the swollen chloroplast proteins. Molotkovsky and Zheskoya (1965) also found that heat induced a three-fold increase in ATPase activity.

Heating resulted in the accumulation of free fatty acids (FFA) in chloroplasts. A suspension of free fatty acids isolated from heated chloroplasts added to a suspension of fresh chloroplasts led to suppression of the Hill reaction. Similar extractions from unheated isolated chloroplasts produced only small amounts of free fatty acids which, when added to a suspension of isolated chloroplasts, did not suppress the Hill reaction. This inhibitory action of FFA on the Hill reaction can be significantly reduced by the addition of ATP, Mg^{+2}, and BSA to the reaction medium.

Unsaturated fatty acids are very effective inhibitors of oxidative phosphorylation in mitochondria (Wojtezak and Lehninger, 1961) and photochemical reactions in chloroplasts (Krogman and Jagendorf, 1959). Heating apparently causes partial destruction of the chloroplast membranes--probably due to the action of lipase--leading to an increase in free fatty acids. Molotkovsky and Zheskoya (1965) suggested that the observed swelling and suppression of the Hill reaction are not the direct result of action by the fatty acids released by the thermal induced lipase destruction of these membrane lipids. Similar swelling and inhibition of the Hill reaction can be brought about by the treatment of fresh suspensions of isolated chloroplasts by the detergent

sodium oleate. This supported the suspected role of free fatty acids as the primary cause of chloroplast injury. The free fatty acids are thought to induce changes in the actiomyosin-like protein of the chloroplast envelopes causing swelling (Molotkovsky and Zheskoya, 1965). The likelihood of such influence of free fatty acids on the teritary structure of proteins was established by Mosolov in 1964 (Molotkovsky and Zheskoya, 1965). Albumin is protective due to its ability to bind free fatty acids.

Working on carbon assimilation by isolated chloroplasts, Walker and Hill (1967) observed that the Hill reaction is more sensitive than previous work by Holt and French (1946) and Arnon and Whatley (1949) suggested. Pea and spinach chloroplasts exposed briefly to 50°C showed progressive enhancement of the Hill reaction with increased exposure time up to an optimum of 30 seconds exposure decreasing to a minimum rate at about 90 seconds. This thermal enhancement of the Hill reaction was explained in terms of uncoupling of photophosphorylation with the electron transport system. It was also noted that the heated chloroplasts showed decreased responses to NH_4Cl which Emmett and Walker (1969) cited as evidence of thermal uncoupling. The physical basis for the uncoupling was interpreted as a brief melting of the lipid components of the chloroplast membranes leading to changes in their permeability properties (Emmett and Walker, 1969).

McClendon (1954) reported that polyethylene glycol (PEG) stabilized chloroplasts and stimulated the rate of the Hill reaction. Clendening (1956) felt that the 30% PEG solution used to stabilize chloroplasts was contaminated with cytoplasmic proteins and they--not the PEG--were the effective agents for the stabilization of chloroplasts. The

protection of chloroplasts from inactivation by heating and aging were also attributed to the protein contaminants.

Data collected by Oku and Tomita (1971), however, led them to suggest that absorption of PEG on chloroplast membranes may protect the membrane structure fom external disturbances such as thermal agitation. However, the elimination of inhibitory substances, such as free fatty acids (Molotkovsky and Zheskoya, 1965) released from membrane lipids following heat treatment, by binding to PEG could well be an important factor which cannot be neglected from any explanation of the observed action of PEG.

Supported by observations by Katoh and San Pietro (1967) and Yamashita and Butler (1968), Oku and Tomita (1971) concluded that PEG protects the Hill reaction from heat inactivation through the stabilization of chloroplast membrane structure by an adsorptive interaction with the membrane.

Hinkson and Vernon (1959) found that incubating chloroplasts at 51^{o}C for 10 minutes resulted in the complete inhibition of the Hill reaction using DPIP as an oxidant. Katoh and San Pietro (1967) studied the effects of heat on Euglena chloroplasts and reported what appeared to be a preferential destruction of only the Hill reaction of photosystem II of the light reaction. The Hill reaction in the presence of ferricyanide was most heat sensitive with a total loss in activity occurring at 40^{o}C. However, these heated chloroplasts could still catalyze the photoreduction of NADP with reduced cytochrome 552 (PSI) while the photoreduction of oxidized cytochrome -552 of PSII was as sensitive to heat as the Hill reaction. Photoreduction of oxidized cytochrome -552 (PSII) dropped to around zero at 40^{o}C while the

photooxidation of reduced cytochrome 552 (PSI) showed a slight increase up to 30°C, then decreased gradually to around 50°C (85% activity) and dropped off to around 25% activity at 60°C.

The irreversible damage to membranes following thermal stress (Heber and Santarius, 1973) can be prevented by the presence of sugars in the surrounding medium during the stress period (Jagendorf and Avron, 1958; Duane and Krogman, 1963; and others). Santarius (1973) observed that heating and freezing of isolated thylakoids in the absence of protective agents causes irreversible inactivation of photophosphorylation and electron transport. Sugar added to the reaction medium prior to thermal stress prevents or reduces the inactivation of these reactions. Less sugar is necessary for the protection of electron transport than for photophosphorylation suggesting that the first effect of thermal stress is uncoupling resulting in stimulation of electron transport. Santarius (1973) found that the protective effect of sugars is a function of both concentration and molecular weight with trisaccharides more effective than dissaccharides and the latter more effective than monosaccharides.

The uncoupling of photophospharylation from electron transport during high temperature stress may be an indicator of one of the primary causes of heat injury (Emmett and Walker, 1969). Heat resistance may be acquired by membrane changes during hardening or perhaps by the accumulation of membrane stabilizing compounds within the cell (Santarius, 1973).

Sugars presumably can influence water binding because of the affinity of their hydroxyl groups to water. The ability of sugar to replace water adjacent to thermal sensitive proteins (Heber and

Santarius, 1964; Huggins, 1965; Santarius, 1968; Parker, 1972) or to bind directly to proteins supports Levitt's theory (1972) that sugar stabilizes membrane proteins against thermal stress. The rise in the thermal resistance in summer may be the result of changes in membrane bound proteins (Alexandrov et al., 1970; Langridge and McWilliams, 1967; Levitt, 1972) or membrane lipids (Molotkovsky and Zhestkova, 1965).

Mukohata (1973) suggested that in any model proposed for thylakoid membranes, lipids play a role to maintain proper spatial relationships among the various components of the thylakoids such as chlorophyll, cytochromes, ATPase, carboxydismutase and others. He postulated that the intermediates of the electron transport chain are inlaid in the lipid of the thylakoid membrane with the sequence of the intermediates determined by the highest probability of oxidation-reduction occurring. This depends on both the redoxpotential and spatial positioning of each component. Coupling of electron transport to phosphorylation would depend on the spatial positioning.

Mukohato (1973) warmed isolated spinach chloroplast suspensions for five minutes at temperatures ranging from 20-55^{o}C. Activities of the chloroplasts as determined by changes in light scattering ($\propto$LS), change in pH ($\propto$pH) and photophosphorylation decrease in parallel when the chloroplasts are warmed above 30^{o}C and lost at 55^{o}C. However, even when the internal structure of the chloroplasts is lost as indicated by $\propto$LS, inactivation of the enzymes and redox components related to the Fecy-Hill reaction are not inactivated. He also suggested that since the activity of the acid-base ATP formation was not lost until warmed over 50^{o}C, the enzymes involved in ATP synthesis might not be fully

inactivated. It therefore seemed most likely that thermal denaturation took place in the lipid component of the membrane with changes in activity occurring due to structural modification of these lipids allowing leakage with concomitant changes in pH and light scattering properties of the chloroplasts (Mukohato, 1973).

Mukohata et al. (1973) suggested that the biochemical site of thermal damage to chloroplast activity may be the pathway common to both photosystems, that is, cytochrome f and/or plastoquinone of the so-called zigzag or Z-scheme. Biophysically, the most probable site of the thermal inactivation is that portion of membrane construction necessary to maintain spatial positioning of the enzymes and/or pigments or perhaps even to maintain the chemiosmotic potential gradient across the membrane.

Red band fluorescence from chloroplast suspensions decreased in apparent response to changes in the integrity of the surroundings of the chlorophyll molecules particularly those of PSII. This was interpreted by Mukohata et al. (1973) to indicate some drastic alterations in thylakoid construction, most likely destruction of the oxygen evolving system.

Since ATPase is inactive in chloroplasts warmed to 55^{o}C and the so-called coupling factor from spinach chloroplast is activated above 60^{o}C (Farron and Racker, 1970), the loss of the acid-base phosphorylation activity after warming chloroplasts as noted might not be caused by naturation of ATPase protein but may be due to lowering of coupling efficiency in the membranes. Inactivation of photophosphorylation could be correlated with inactivation of electron transport.

Inactivation of phosophorylation may be attributable to the

decrease in coupling efficiency (uncoupling) possibly due to alteration in membrane structures which may cause increased rates of substrate transport or increased electron transport after the electron transport mechanism is short circuited (Mukohata, 1973) resulting in stimulation of electron transport without photophosphorylation. Further increases above the maximum permissible temperature (45°C) apparently induce alterations which are fatal to photosynthetic activities and the membrane structure itself (Mukohata et al., 1973).

Mukohata et al. (1973) concluded that thermal inactivation of photophosphorylation in isolated chloroplasts is due to a loss in coupling efficiency between the electron transport system which can be attributed to changes in the lipid portion of the thylakoids.

Exposure of isolated spinach thylakoids to 40°C caused an initial stimulation of DCIP (dichlorophenolindophenol) reduction and immediate reduction in cyclic photophosphorylation (Santarius, 1973). The stimulation of electron transport, as observed in both cases, along with the decrease in photophosphorylation may be considered as uncoupling effects (Mukohata et al., 1973; Emmett and Walker, 1969, 1973). Spring harvested chloroplast demonstrated no significant differences in thermostability of electron transport and photophosphorylation probably due to an increased thermal sensitivity by DCIP reduction and decreased heat sensitivity of cyclic phosphorylation. With extended heat treatment or higher temperatures, cyclic photophosphorylation of spring or summer chloroplasts became even less inactivated than DCIP reduction (Santarius, 1974). This may be partially explained by the high sensitivity of the water splitting apparatus plus the stability of

photosystem I (Katoh and San Pietro, 1967; Yamashita and Butler, 1968). These changes in thermosensitivity following hardening and dehardening are apparently due to changes in membrane proteins or lipids either of which could influence the thermostability of these biomembranes (Langridge and McWilliams, 1967; Alexandrov et al., 1970; Levitt, 1972).

In 1975, Santarius once again attempted to further elucidate the effects of heat on living cells by comparing the thermostabilities of various biochemical reactions of isolated chloroplasts. Thermal inactivation of four different water soluble chloroplast stroma enzymes were measured. Inactivation of DCIP reduction by these sema chloroplasts was determined for comparison. Data showed that the enzymes became inactivated at much higher temperatures than the photosystem II dependent electron transport reaction indicating that chloroplast membranes are more heat sensitive than individual enzymes. Furthermore, he demonstrated that photosystem I dependent oxygen uptake is much more resistant than photosystem II dependent DCIP reduction. Photosystem I dependent oxygen uptake during methylviologen reduction increased markedly (up to 22.5%) up to 50°C and dropped rapidly to zero activity at about 70°C. By contrast, photosystem II dependent DCIP reduction dropped off rapidly to about zero activity at about 48°C. The stimulation of photosystem I reduction at moderate temperatures was interpreted as the result of thermal induced membrane alterations. Santarius (1975) felt that the thermal inactivation of photosystem II is at least partially the result of the inhibition of the water splitting site. The observed slight stimulation of electron transport in DCIP reduction between 30°-35°C apparently supports thermal uncoupling as described by Emmett and Walker (1969, 1973) and Mukohata et al.

(1971). Data clearly showed that non-cyclic photophosphorylation is the most heat sensitive membrane reaction (Santarius, 1975).

Packed volumes of chloroplast membranes following centrifugation aid in the collection of information about permeability properties of chloroplasts before and after temperature treatment. Decreased chloroplast volumes suggest increased envelope permeability resulting in leakage while increased volumes suggest alterations in membrane structure resulting in swelling. Thylakoids heated for three minutes at 90°C were much more compressed than controls at 30°C while those treated at 49°C showed a higher packed volume. These data suggest that thylakoid treated at 90°C lost permeability properties while those treated at 49°C were swollen.

Membranes of these swollen chloroplasts still retained their permeability properties but had almost completely lost their electron transport mechanisms in photosystem II and cyclic and non-cyclic photophosphorylation. This suggests that the inactivation of non-cyclic photophosphorylation is not due simply to an increase in permeability of the chloroplast membranes during heating but to other membrane alterations (Santarius, 1975) most likely the lipid moiety (Mukohata et al., 1973). Molotkovsky and Zhestkova (1965) suggested that heating induced lipase hydrolysis of lipids producing free fatty acids which induced membrane alteration necessary for the various biochemical reactions associated with them.

Santarius (1975) also suggested that the membrane bound ATPase responsible for the terminal energy conservation step in photophosphorylation might be heat sensitive and probably plays a role in the thermal inactivation of photophosphorylation. Experimental data

indicated that the ATPase, sometimes referred to as the coupling factor CF, could be removed from the thylakoids by washing with 0.75 mm EDTA. The inactivation of cyclic photophosphorylation by heating EDTA washed thylakoids and the reconstitution of this system by adding non-heated coupling factor occurred to the same degree as the inactivation of controls which had retained the coupling factor (i.e., had not been washed with EDTA). Inactivation of cyclic photophosphorylation did not occur when heated ATPase was added to the EDTA--washed thylakoids suggesting that cyclic photophosphorylation is more heat sensitive than the coupling factor under these experimental conditions.

Vambutas and Racker (1965), Lynn and Straub (1969) and Lien and Racker (1971) reported the activation of membrane bound ATPase by trypsin. When heat treatment of thylakoids was followed by trypsin digestion and activation of ATPase by Ca^{+2} results were similar to those observed when isolated ATPase was heated (Santarius, 1975). At the same time, activity of membrane bound ATPase requiring light, thiols, and Mg^{+2} for activation (Marchant and Packer, 1963) became inactivated by heat almost to the same extent as cyclic photophosphorylation.

At first glance, the thermal inactivation of this light, Mg^{+2} and thiol-requiring ATPase might not appear to fit into a concept which explains heat damage by membrane alterations beyond the protein level. After more thorough study of this phenomenon, however, one realizes that the light requirement for ATPase activity indicates that the splitting of ATP requires a special membrane configuration which must be induced by a high energy induced condition of the membrane. Also, the stimulation of ATPase activity by trypsin digestion made the thermosensitivity of the enzyme responsible even less apparent,

agreeing with the observation that the coupling factor (CF_1) was much less heat sensitive than the membranes whether exposed to heat while it was attached to the chloroplast membranes or detached from them (Santarius, 1975). Present interpretation is that the coupling factor (CF_1) incorporates the same enzymatic activity as the above mentioned ATPase catalyzing ATP formation if in the presence of a suitable thermodynamic gradient, while the ATPase activities represent the exergonic back reaction.

Thus, we are as yet unable to explain the different sensitivities of cyclic and non-cyclic photophosphorylation. There is, of course, wide agreement as to the existence of two native coupling sites within the electron chain in thylakoid membranes (see Santarius, 1975). According to the chemiosmotic theory of coupling, the coupling sites for photophosphorylation may be identified with sites liberating protons into the inner space of the thylakoid membrane vesicles during electron transport. It is possible that the coupling site closest to the water splitting system is particularly sensitive to heat because of the observed high sensitivity of the water splitting apparatus. This could explain the observation that non-cyclic photophosphorylation of PSII is always the most heat sensitive membrane reaction irrespective of the physiological state of the membrane. In contrast to PSII, PSI is comparatively heat stable.

One must recognize the similarity between heat effects on chloroplasts membranes and the effect of freezing (Heber and Santarius, 1964; Heber, 1967; Heber et al., 1973; and others). It is unlikely that this is a simple coincidence. It is more likely supportive evidence pointing to membrane as the common stress sensitive site

within the cell. Experimental data, however, points to different membrane alterations for inactivation due to freezing and heat stress (Santarius, 1975).

Krause and Santarius (1975) explored the thermosensitivity of the double membrane chloroplast envelope which enclosed the thylakoid imbedded in the semiliquid stroma. These two membranes possess different permeability properties and probably have different compositions. It is reasonable to assume that alterations of its properties could induce altered permeability properties and possibly disruption of normal chloroplast activity.

Initial experiments demonstrated that thylakoids enclosed in the envelopes are less heat sensitive than envelope free thylakoids. However, pH of the reaction medium plays a role in the heat sensitivity of the intact chloroplasts since any pH changes are transmitted to the chloroplast stroma. Intact chloroplast incubated at $47^{o}C$ for 3 minutes showed 50% inactivation of ferricyanide reduction by the thylakoids if the pH was between 6 and 7. Higher and lower pH values induced higher levels of heat sensitivity. Krause and Santarius (1975) avoided pH related complications of their data on heat sensitivity by performing all experiments at a pH = 6.7.

Intact chloroplasts incubated three minutes at $50^{o}C$ showed 70% irreversible inhibition of photophosphorylation and light-dependent ferricyanide reduction accompanied by drastic changes in light-induced H^{+} uptake into the thylakoids and altered chlorophyll fluorescence characteristics. These observations indicate severe damage to the thylakoid membranes and comparatively little damage to the integrity of chloroplast envelopes.

The effects of temperature on chloroplast envelope integrity were demonstrated using the activity of certain stroma enzymes. These enzymes do not respond to certain external substrates as long as the chloroplast envelopes remain intact. NAD dependent malate dehydrogenase was only partially inactivated and aldolase almost completely unaffected by temperatures that strongly inactivated thylakoid associated photochemical reactions. Their data suggested that only temperatures of $50^{o}C$ and above decreased the activity of the stroma enzymes suggesting heat disruption of envelope membrane integrity. These results were confirmed by comparison of the visual appearance of the chloroplasts under phase optics. Only a small percentage of the chloroplasts showed disruption of chloroplast envelopes following exposure to $50^{o}C$ for three minutes.

The envelope is essential to the translocation of various substrates and metabolites of carbon reduction and other metabolic processes in the stroma. Since soluble enzymes are relatively heat stable (Santarius, 1975), thermal inactivation of many of the biochemical reactions may be due to disruption of the chloroplast envelopes. Thermal stress does not seem to significantly impair the transport function of the envelopes since the translocators did not seem to exhibit a higher thermal sensitivity than the thylakoid reactions. The envelope continued to function as a barrier to stroma enzymes and certain cofactors and substrates.

Krause and Santarius (1975) concluded that their tests indicated low heat sensitivity of the chloroplast envelopes. This is especially interesting if we remember that the envelopes are more sensitive to brief ultrasonic treatment or osmetic stress. Short periods of

freezing also resulted in loss of chloroplast envelopes without apparent damage to the thylakoids, supporting the conclusion that heating and freezing have different sites of damage (see Santarius, 1975). This behavior of chloroplast envelopes following heat stress may be due to their lipid composition which is reportedly significantly different from that of thylakoids as reported by Douce et al. (1973), Poincelot (1973), MacKender and Leech (1974), and others.

The increased heat susceptibility of light-dependent ferricyanide reduction in envelope free chloroplasts compared to intact chloroplasts supports the conclusion that the envelopes are more heat resistant than the thylakoid membranes and provide a measure of protection to the thylakoids against heat stress (Krause and Santarius, 1975).

Gerwick et al. (1977) studied the effects of supra-optimal temperatures for the function of chloroplasts isolated from the cactus Opuntia polyacantha. Electron transport in Opuntia was found to function at higher temperatures than photophosphorylation. Electron transport, as measured by oxygen evolution and their reduction of either ferricyanide or NADP, was shown to have a temperature optimum between 37°C and 42°C while photophosphorylation with ferricyanide or NADP as the electron acceptor had a temperature optimum of 35°C. These reactions showed about a three-fold increase between the lowest (15°C) and the respective temperature optima except ferricyanide reduction which demonstrated a four-fold increase between 15°C and 40°C. This data conflicts with previous data showing photophosphorylation to be more thermal sensitive than electron transport (Santarius, 1974; Mukohata et al., 1973; Krause and Santarius, 1975; and others). Stimulation of

electron transport at high temperature was interpreted as a result of thermal uncoupling (Emmett and Walker, 1969; Krause and Santarius, 1975).

Gerwick et al. (1977) also found a differential coupling of electron transport to phosphorylation ($P/_2e$) with different electron acceptors. The $P/_2e$ value decreased with temperature when ferricyanide was the electron acceptor but remained close to unity up to about 35°C above which uncoupling occurred when NADP was used as the electron acceptor. These results were interpreted to reflect a relative increase in reduction of ferricyanide of PSII (Trebst, 1974) as the chloroplast membranes were heat damaged.

The observed temperature responses of oxygen evolution (the Hill reaction) and ferricyanide and NADP reduction indicated an optimum temperature for electron transport in isolated cactus (O. polycantha) chloroplasts of 40°C which is higher than the temperature optima reported for these photochemical reactions in other units.

Gerwick et al. (1976) interpreted this 40°C temperature optimum for electron transport and the 35°C optimum for photophosphorylation in isolated Opuntia chloroplasts as indicative of the photochemical energy production necessary for CO_2 fixation at high temperature in Crassalacean Acid metabolism. The drop in these photochemical reactions above the temperature optimum suggests alterations in the thylakoid membranes.

Evidence has been cited in this literature review indicating that the integrity of the chloroplast envelopes as well as the composition of the medium they are suspended in may significantly affect the apparent thermal stability of the isolated chloroplasts. These results

further suggest that studies with isolated chloroplasts may not show the same thermal stability as the intact tissue (Pearcy et al., 1977).

Pearcy et al. (1977) cited data indicating that six minutes at 46°C caused almost as much damage to isolated chloroplasts as twenty-five minutes exposure at 46°C given to the whole leaf twenty-five minutes prior to chloroplast isolation. Preliminary experiments showed that chloroplasts from the high temperature grown plants were also rapidly inactivated. These differences induced Pearcy et al. (1977) to further examine the effects of heat on intact tissues.

Results showed that chloroplasts isolated from Atriplex grown at 23°C/18°C and exposed to varying periods of treatment at 46°C showed marked decreases in reduction of PSII DCPIP reduction with increasing exposure time while isolated chloroplasts from 43°C/30°C grown plants showed no apparent decrease in DCPIP reduction when exposed to up to 40 minutes of heat treatment at 46°C. Thus higher growth temperatures apparently increased thermal stability of photosystem II.

Data also showed that increased thermal stability of light-saturated CO_2 fixation is correlated with increased thermal stability of the quantum efficiency of CO_2 uptake and the light-harvesting reactions of the photosynthetic apparatus as indicated by both in vitro and in vivo measurements of thermal stability of PSII (Pearcy et al., 1977).

Much evidence seems to point to photosynthesis--especially the light absorbing reactions--as perhaps the most thermal labile steps in cellular metabolism of plants (Pearcy et al., 1977). Direct comparisons have shown that photosynthesis is inhibited at temperatures several degrees below the temperature necessary for inhibiting protoplasmic

streaming (Alexandrov, 1964), and respiration (Bjorkman, 1975). Direct comparative measurements have also shown that the soluble enzymes of the chloroplast stroma are more thermal stable than the membrane-dependent light harvesting apparatus as previously discussed (Krause and Santarius, 1975).

Pearcy et al. (1977) examined two approaches to assaying the integrity of PSII in the intact tissue as supplements or alternatives to studying PSII activity in isolated chloroplasts. The first assay involved light induced absorption changes that reach a maximum in both isolated chloroplasts and leaves around 515nm. This observation provides a sensitive indicator of shifts in chlorophyll absorption spectra as well as caratenoids and have been attributed to the establishment of an electrical field across the thylakoid membranes (Witt, 1971). Experimental results showed that Atriplex plants grown at 43°C had a much smaller change in absorbance after exposure to 47°C reaching a maximum change of about 20% after about three minutes and leveling off for up to 17 minutes. Plants grown at 25°C were found to be much more heat sensitive showing a rapid drop in absorbance of about 65% from the initial absorbance after about 3 minutes dropping off gradually to only 10% of the initial absorbance after 17 minutes, a change of 90%. This clearly showed that temperature induced in vivo absorbance chances are much less dramatic in leaves grown at high temperatures than those grown at low temperatures indicating a higher in vivo thermal stability of PSII in high temperature grown plants (Pearcy et al., 1977).

A second approach to assaying the integrity of PSII involves chlorophyll fluorescence from intact leaves. This fluorescence reflects the oxidation and reduction of the primary electron acceptor "Q"

in PSII and thus provides an indicator of electron transport (Duysens and Sweers, 1963). The increased fluorescence upon illumination of the leaf is characterized by a rapid increase in the initial level of fluorescence (F_o) followed by a slower increase as Q is reduced. In the presence of DCMU, which blocks the oxidation of Q by the electron transport system, fluorescence rises rapidly (measured in 0.1 seconds) to a maximum fluorescence level (Fmax). Experimental data showed that increasing the temperature 1°C/minute for Atriplex grown at 45°C/30°C resulted in no increase in the FO-Fmax ratio up to about 48°C, then sharply up to around 51°C. In contrast, plants grown at 20°C/15°C show no increase up to around 37°C, rose gradually up to around 40°C, then sharply up to around 45°C tapering off gradually to a maximum Fo/Fmax at around 47°C. If we can assume that these shifts reflect the thermal stability of PSII in vivo, then we can conclude that growing the plants at higher temperature induced a 5°-8°C upward shift is stability of PSII (Pearcy et al., 1977).

Thus the data presented here clearly suggested that the stability of photophosphorylation and PSII electron transport result from increases in growth temperature suggesting the possibility that common mechanisms are involved in the heat inactivation and observed changes in heat stability of both processes. The apparent localization of PSII in the thylakoid membranes plus the similarity of action of heat treatment and lipid specific reagents (Mukohata, 1973) suggested to Pearcy et al. (1977) that properties of the thylakoid membrane system--particularly the membrane lipids--may play an important role in determining the heat stability of these processes.

Schreiber and Armond (1977) investigated the heat induced changes in the photosynthetic pigment system by analyzing the effects of heat on the chlorophyll fluorescence excitation and emission properties of chloroplasts isolated from Larrea divaricata. This plant is a desert shrub which exhibits a high resistance to heat and water stress.

Schreiber and Armond (1977) observed a heat-induced rise in chlorophyll fluorescence which was correlated with heat damage to chloroplasts. The presence of chlorophyll in the thylakoid membranes led to the conclusion that heat alters the properties of thylakoid membranes, inducing changes in fluorescent characteristics and the activities of membrane-bound enzymes. Increased growth temperatures apparently leads to adaptations by the plant to these higher temperatures with concomitant alterations in thylakoid membrane composition. Schreiber and Armond (1977) suggested that phase changes would shift the heat-induced fluorescence and enzyme damage to higher temperatures. The authors felt that the observed heat-induced fluorescence could not be explained strictly on the basis of heat-induced alteration in thylakoid membrane lipid fluidity as suggested by Murata et al. (1975) nor by the heat-induced blockage of photosystem reaction centers reported by Schreiber et al. (1976).

The significant differences in fluorescence emissions suggested to Schreiber and Armond (1977) that heat interferes with the transfer of energy between chlorophyll a and b. Unheated chloroplasts showed no chlorophyll b fluorescence indicating 100% energy transfer to chlorophyll a while heat treated chloroplasts produced sizeable chlorophyll b fluorescence indicating a heat induced block in energy transfer from chlorophyll b to chlorophyll a representing a new aspect

of heat damage at the pigment level (Schreiber and Armond (1977).

Armond et al. (1977) proposed a model for the photosynthetic unit in which the light harvesting complex is associated with photosystem II (PSII). Mg^{+2}, which is required for PSII, was purported to regulate the degree of interaction between the light harvesting complex and PSII (Davis et al., 1976). Findings by Schreiber and Berry (1977) suggested that depletion of Mg^{+2} enhances heat-induced fluorescence changes which may indicate that heat treatment of chloroplasts leads to disassociation of the light harvesting complex from PSII.

Schreiber and Armond (1977) suggested that heat-induced fluorescence changes are determined by (1) increased chlorophyll a-fluorescence following the block of PSII centers, and (2) decreased chlorophyll a-fluorescence following blockage of the transfer of excitation energy from chlorophyll b to chlorophyll a or from the light harvesting complex to the core complex. The proportion of light absorbed by chlorophyll b (or within the light-harvesting complex) relative to chlorophyll a (or within the core complex) will determine the extent of the increase of heat-induced fluorescence.

CHAPTER III

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of cabbage (Brassica oleracea, c.v. Early flat Dutch), corn (Zea mays, L. cv. I. O. Chief), milo (Sorghum bicolor cv. Funks hybrid 522), oats (Avena sativa L. Holden), soybean (Glycine max L. cv. Brysoi 9), tomato (Lycopersicon esculentum L. cv. stone), and wheat (Triticum aestivum L. cv. Arthur) were sown in flats (42.5 cm. x 28 cm. x 5 cm.) containing perlite and vermiculite (1:1). The plants were grown in two Sherer model CEL 37-14 environmental chamgers at constant day/night temperatures of 20°C or 31°C. The growth chambers were programmed for sixteen hours of light and eight hours of darkness with the light intensity increasing in the morning and decreasing in the evening to simulate normal environmental conditions. Each growth chamber contained eight 100-watt incandescent bulbs and sixteen mixed Sylvania soft-white and Gro-lux fluorescent tubes with a maximum illumination of approximately 1600 foot-candles at plant height. Plants were watered daily and fed twice a week with 750 cc of a complete nutrient solution (Kaufman et al., 1975) per flat.

Effects of Temperature on the Growth Rate

One hundred seeds of corn, milo and wheat were grown at 20°C and

31°C. The heights of 30-40 plants at each treatment were measured and recorded at selected intervals over a 28-day period. The mean heights of each species grown at each temperature were determined at each interval.

Isolation of Chloroplasts

Chloroplasts were isolated from 5-10 gram of leaf tissue by modifications of the methods of Leech (1966) and MacKender and Leech (1974). The deveined leaves were cut into small pieces into a Waring blender containing 70 milliliters of ice-cold 0.3M sucrose in M/15 Sorensen phosphate buffer (pH 6.8) and homogenized by turning the blender on high for 5 seconds then off for 5 seconds. This on-off procedure was continued for one minute of grinding time.

The homogenate was filtered through one layer of nylon hose which was squeezed to remove as much crude filtrate as possible from the macerated leaf tissue. This removed most of the leaf fragments. The crude filtrate was filtered through eight layers of nylon hose three times (do not squeeze) to yield the crude chloroplast suspension.

The filtrate was centrifuged at 300xg for 15 seconds at 4°C in a Sorvall RC2-B superspeed refrigerated centrifuge using a Sorvall 50 ml capacity swinging bucket head. The pellet was collected, resuspended in 10 ml of cold buffer in 0.3M sucrose and layered on top of 10 ml of phosphate buffer in 0.4M sucrose (pH 6.8). These discontinuous sucrose gradients were centrifuged at 500xg for 12 minutes. The pellet consisted primarily of intact chloroplasts as determined under phase contrast microscopy using a Wild Heerbrugg M20 phase microscope under 400x magnification. Intact chloroplasts appear bright green with

bright halos while ruptured chloroplasts appear gray and lack the halos.

It was very important that all manipulations be done at low temperatures. Nitrogen gas was bubbled through the suspension as often as possible to reduce the chances for oxidation of unsaturated fatty acids.

Effects of Supra Optimal Temperatures on Chloroplast Membrane Permeability

Isolated chloroplasts were resuspended in 75 ml of buffer solution in 0.3M mannitol and transferred to a graduated cylinder. The cylinder containing the chloroplast suspension was immersed in a variable temperature water bath (Fisher Serological Bath or Precision Scientific Thelco Model 83). A conductivity cell (YSI 3401, or 3403; K=1.0) and a thermometer were placed in the chloroplast suspension. After the temperature of the chloroplast suspension reached equilibrium, the conductance was measured using either a YSI Model 31 or a Barnstead Model PM70cB conductivity bridge. Following the initial reading at room temperature, the temperature of the chloroplast suspension was increased slowly at a rate of about $1^{o}C$ every three to five minutes and the conductance recorded for each degree increase. Conductivity was calculated by multiplying conductance by the cell constant (K=1.0). Conductance determinations were repeated 2-3 times per growth temperature per species.

Microscopic Evaluation of Heated Chloroplasts

Slides were made of the chloroplast suspension before and

periodically during heat treatment and observed using a Wild Heerbrum M20 phase microscope under 400x magnification. Changes in chloroplast morphology were observed and recorded.

Lipid Extraction and Isolation

The chloroplast pellet was dried under N_2 and resuspended in 5 ml of methanol. The chloroplasts in methanol suspension was heated under N_2 at 55°C in a water bath to break the lipids away from the proteins in the membranes being careful not to let all of the methanol evaporate. The chloroplast in menthanol suspension was transferred to a 15 ml glass vial and enough chloroform added to make a 2:1 chloroform-menthanol suspension with a total volume of 15 ml.

A strip of teflon tape was placed over the mouth of the vial and a cap screwed down loosely. The vial was placed in an oven preheated to 35°C. After 15 minutes, N_2 was again bubbled through the suspension and the cap was screwed down tightly. The vial was left in the oven overnight (about 15 hours).

The suspension was transferred to a test tube and fifteen ml of 2M KCl was added and mixed thoroughly by bubbling N_2 through the suspension. After standing a short period of time, the suspension separated into two layers with the chloroform and lipids on the bottom. The upper layer was aspirated and discarded; 2M KCl was again added and mixed thoroughly under N_2. The upper layer was aspirated and discarded. The chloroform suspension remaining was washed twice with 10 ml of distilled water which was aspirated off and discarded.

The chloroform suspension was passed through a column of anhydrous Na_2SO_4 to remove all water, collected in a clean, weighed

vial, and dried under N_2 in a water bath at 35°C. The vial containing the lipids plus large quantities of pigments--especially chlorophyll--was reweighed. The weight of the contents was determined by subtracting the weight of the vial.

The lipid-pigment extract was stored in the freezer at -20°C until analyzed.

Separation of Lipids by Thin Layer Chromatography

Twenty-five grams of silica gel G (Acc. to Stahl) was mixed with 50 ml of ethanol and water (1:1) to form a homogeneous slurry. Glass TLC plates (20 cm x 20 cm) were coated with the silica gel G slurry 250 microns thick using a Desaga Brinkman spreader. After drying, the plates were stored in a Brinkman storage cabinet over anhydrous $CaSO_4$ until used.

Immediately prior to use, the TLC plates were activated for 30 minutes at 110°C.

The lipid extract was resuspended in 100 ul of chloroform. Twenty microliters of the lipid extract were spotted at the lower corner of the activated plates, dried under N_2, and transferred to the first solvent tank.

Two dimensional thin layer chromatography was used to separate the lipid species, according to the method of Schwertner and Biale (1973). Brinkman developing tanks (23 cm x 29.5 cm x 9.5 cm) were prepared by thoroughly cleaning and washing three times with pure acetone to remove all traces of oil or grease. A 20 cm x 57 cm piece of Whatman #1 chromatography paper was and placed into the developing tanks to ensure

saturation of the internal atmosphere of the tank by the solvent system. The solvent system (approximately 100 ml) was poured slowly over the paper and down the sides of the tank. The tanks were covered with heavy glass plates and permitted to equilibrate (usually at least one hour). The tanks were cleaned and new solvent systems prepared frequently to ensure reproducibility.

Twenty microliters of lipid extract were transferred to the TLC plate, dried under N_2 and placed in the developing tank containing chloroform-methanol-water (65:25:4), hereafter referred to as solvent system 1. After the front had moved to within approximately one centimeter of the top edge of the plate, the plate was removed from solvent system 1 and dried under N_2. The front was measured and the silica gel scraped off to remove any impurities which could interfere with the separation of the second solvent system. The plate was rotated 90°C and immersed in acetone-acetic acid-water (100:2:1), hereafter referred to as solvent system II, which effectively separated the galaitolipids and sulfolipids from the phospholipids. The addition of acetic acid to the acetone helped prevent trailing, the loss of neutral lipid fatty acids, and contamination by other lipids (Schwertner and Biale, 1973).

When the mobile phase of the solvent system reached to approximately one centimeter from the top, the plate was removed, dried under Na, and visualized in iodine.

The visualized lipid spots were outlined using a clean scribe before the color faded. Measurements were made and Rf values calculated for each solvent system. The different lipid species were identified using standards and Rf values published by Schwertner and Biale,

1973. (see Figure 1). The spots were scraped from the plates into clean, oil-free 15 ml. vials.

Transesterfication of Lipids

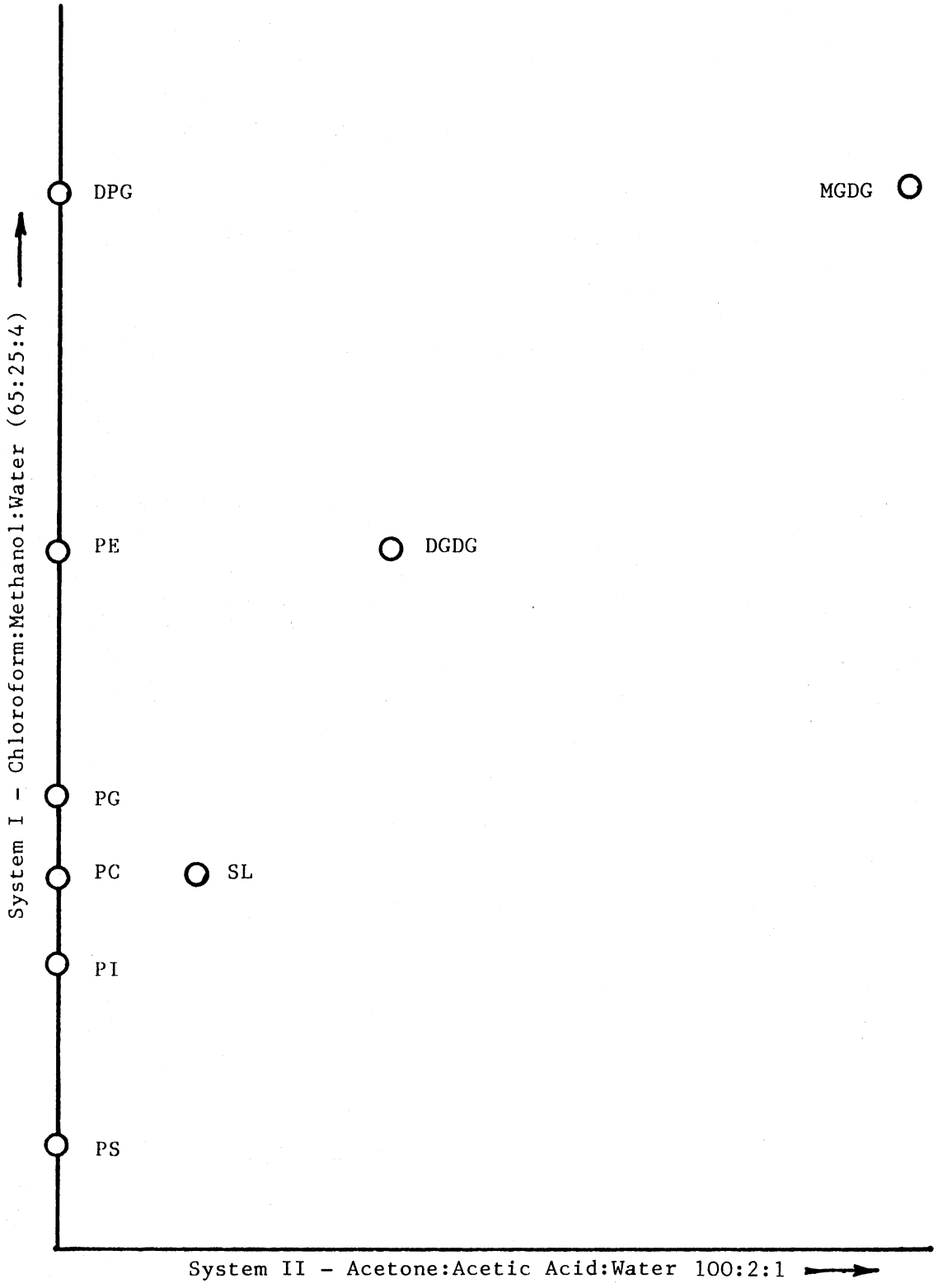
Transesterfication of lipid fatty acids was accomplished by adding 0.5 ml of 2:2--dimenthoxypropane (a water scavenger)(personal communication with Dr. G. Odell) and 0.5 ml of 5% HCl in lipopure methanol (Applied Science Laboratories). After bubbling N_2 through the solution, the vials were loosely capped and placed in an oven at 50°C for 10 minutes. N_2 was again bubbled through the solution. The mouth of the the vial was covered with teflon tape, the cap was screwed down tight, and the vials incubated over night in the oven at 50°C (12-15 hours).

Following transesterfication, an equal volume of deionized water was added followed by 3 ml of n-hexane. The mixture was mixed by vigorously bubbling N_2 gas through the mixture. The upper hexane layer containing the fatty acid methyl esters was drawn off with a Pasteur pipette and dried by passing it through an anhydrous Na_2SO_4 column. The effluent was collected in a clean vial. This procedure was repeated three times to remove all fatty acid methyl esters from the aqueous layer. This drying column was made by packing glass wool in the bottom of a Pasteur pipette and pouring granular anhydrous Na_2SO_4 into the pipette up to within 1-2 cm from the top (approximately 8 cm of Na_2SO_4). The Na_2SO_4 column was washed with 5 ml. of n-hexane which was also collected in the vial.

The n-hexane fatty acid mixture was evaporated under N_2 in a water bath heated to 45°C. The fatty acid methyl esters were resuspended in 25 ul of carbon disulfide for injection onto the gas

Figure 1. Two Dimensional Thin Layer Chromatographic Separation of Chloroplast Lipids

Code:
Phosphatidylserine (PS)
Phosphatidylinositol (PI)
Phosphatidylcholine (PC)
Sulfoquinovosyldiglyceride (SL)
Phosphatidylglycerol (PG)
Phosphatidylethanolamine (PE)
Digalactosyldiglyceride (DGDG)
Diphosphatidylglycerol (DPG)
Monogalactosyldiglyceride (MGDG)


DPG
MGDG
PE
DGDG
PG
PC
SL
PI
PS
System I - Chloroform:Methanol:Water (65:25:4)
System II - Acetone:Acetic Acid:Water 100:2:1

chromatograph. Carbon disulfide gives a smaller solvent peak than hexane and a better base line with a hydrogen flame detector (Allen and Good, 1971).

Gas Chromatography and Quantitation of Lipids

The concentrated fatty acid methyl esters were chromatographed on a Beckman GC-4 gas liquid chromatographer with a matched pair of 6 foot x 2 mm ID glass columns containing 15% diethyleneglycol succinate (DEGS) on chromosorb W (HP) 100/120 mesh. Separation was accomplished with a column temperature of 175°C (isothermic detector temperature of 250°C, and a helium flow rate of 40 ml/minute.

Injection of samples were made using the solvent-flush technique. Approximately 2 ml of solvent (CS_2) was drawn into the syringe. A small air pocket was formed in the needle end of the syringe of avoid mixing of the sample and solvent. The desired quantity of sample was then drawn into the syringe. The needle was removed from the sample solution and the plunger pulled back until the entire sample was visible with an air pocket on both sides. The exact sample size was read using this technique and was ready for injection. The solvent behind the sample ensured that the entire sample was injected into the column minimizing error.

The retention time of each methyl ester was determined using standards obtained from Sigma Chemical Company and data reported by Allen et al. (1966). The retention time of each fatty acid was calculated relative to palmitic acid using three runs of each fatty acid methyl ester. (Table I) Absolute retention times are unreliable, shifting

TABLE I

RETENTION TIMES OF FATTY ACID METHYL ESTERS
RELATIVE TO PALMETIC ACID (16:0) ON
15% DEGS COLUMN

Fatty Acid		Relative Retention Time
Palmitic Acid[a]	16:0	1.0
Palmitoleic Acid[a]	16:1	1.2
Hexadecatrienoic Acid[b]	16:3	2.6
Heptadecanoic Acid[a]	17:0	1.4
Stearic Acid[a]	18:0	1.85
Oleic Acid[a]	18:1	2.2
Linoleic Acid[a]	18:2	2.8
γ-Linolenic Acid[a]	18:3	3.5
α-Linolenic Acid[b]	18:3	4.1
Arachidic Acid[a]	20:0	3.3
11-Eicosaenoic Acid[a]	20:1	3.8
11,14-Eicosadienoic Acid[a]	20:2	4.8
11,14,17-Eicosatrienoic Acid[a]	20:3	6.5

[a]Retention times determined with standards from Sigma

[b]Retention times obtained from Allen et al., 1966

slightly with changes in injection volume, column temperature, carrier gas flow rates and other factors.

The lipid fatty acids were quantitated by multiplying the peak height by the peak width at one half peak height. This was facilitated by the use of a peakometer (Alltech Associates). Measured peak areas were converted to a molar quantity (mole adjusted peak area) by multiplying the peak area by a proportionality factor (Table II). These molar adjustment factors were calculated by dividing the molecular weight of each fatty acid by the molecular weight of the internal standard, heptadecanoic acid (Allen and Good, 1971). The fatty acid composition of each lipid was expressed as the mole percent of that lipid.

The chloroplast lipids were quantitated by the method of Allen and Good (1971) using the equation:

$$\text{nanomoles of lipid} = \text{nanomoles of standard} \left(\frac{\text{sum of adjusted peak area}}{\text{peak area of standard}}\right)$$

where

n is the number of acyl residues in the lipid molecule. The nanomoles of lipid was adjusted for each lipid species to the quantity per microliter of mixture injected and to the constant attenuation of 1000. The mole percent of each lipid species was calculated using these adjusted values.

These data were compiled from two samples of leaves from each species analyzed. Lipid analyses were repeated three times per sample.

TABLE II

MOLECULAR WEIGHTS AND MOLAR CORRECTION FACTORS OF FATTY ACID METHYL ESTERS RELATIVE TO METHYL HEPTA DECANOATE (17:0)

Fatty Acid	Molecular Weight of Methyl Ester	Molar Correction Factor[a]
Methyl Palmitic Acid (16:0)	270.5	1.05
Methyl Palmitoleic Acid (16:1)	268.5	1.06
Methyl Hexadacatrienoic Acid (16:3)	264.5	1.08
Methyl Heptadecanoic Acid (17:0)	284.5	1.00
Methyl Stearic Acid (18:0)	298.6	0.953
Methyl Oleic Acid (18:1)	296.5	0.960
Methyl Linoleic Acid (18:2)	294.5	0.966
Methyl γ-Linolenic Acid (18:3) (6,9,12 - Cis, Cis, Cis-Octadecatrienoate)	292.5	0.973
Methyl α-Linolenic Acid (18:3) (9,12,15 - Cis,Cis, Cis-Octadecatrienoate)	292.5	0.973
Methyl Arachidic Acid (20:0)	326.6	0.871
Methyl-11-Eicosaenoic Acid (20:1)	324.6	0.877
Methyl-11, 14-Eicosadienoic Acid (20:2)	322.5	0.882
Methyl-11, 14,17-Eicosatrienoic Acid (20:3)	320.5	0.888

[a]Calculated relative to methyl heptadecanoate

CHAPTER IV

RESULTS

The Effects of Temperature on Growth

Corn, wheat and milo were grown at 20°C and 31°C. Germination and growth rates were recorded over a 28-day period (Table III). Corn and milo appeared to be less sensitive to the higher growth temperature (31°C) than did wheat (see Figure 2). Both corn and milo had the same growth pattern (Figures 3 and 4), although corn grew at a faster rate than milo at both temperatures. Initially, both corn and milo grew faster at 31°C than at 20°C. However, at the later stages, the plants grown at 31°C seemed to catch up and grow at similar rates.

The growth pattern of wheat was quite different. Plants grown at 20°C and 31°C seemed to have about the same growth pattern until the 24th day where plants growing at 31°C began to grow at a slower rate (Figure 2). Wheat grown at 20°C appeared to continue growing at a constant relative growth rate through the 28th day of this experiment.

The Effects of Temperature on Chloroplast Membrane Integrity as Determined by Changes in Conductivity

The results of this experiment are summarized in Table IV and Figure 5. (A sample Table V and graph, Figure 6, are included).

TABLE III

GROWTH OF PLANTS AT 20°C OR 31°C

Days of Growth	Wheat		Milo		Corn	
	20°C	31°C	20°C	31°C	20°C	31°C
0[a]						
4		0.9			0.9 ± .07	1.52 ± .42
5	1.3 ± .21	1.5 ± .62		1.2 ± .6	0.72 ± .37	2.96 ± 1.64
6	2.85 ± 1.34	3.64 ± 1.41	0.41 ± 0.4	2.44 ± 1.01	0.95 ± .58	6.29 ± 2.86
8	4.88 ± 3.23	6.7 ± 3.41	0.86 ± 0.6	6.38 ± 2.76	2.11 ± 1.4	11.33 ± 4.35
10	7.3 ± 3.93	10.4 ± 3.60	1.67 ± 1.54	9.72 ± 3.50	4.20 ± 2.66	15.61 ± 5.72
13	12.85 ± 5.83	17.19 ± 6.51	3.14 ± 2.9	15.11 ± 5.37	7.82 ± 4.49	21.83 ± 7.01
17	19.69 ± 7.11	21.92 ± 4.7	6.06 ± 5.0	18.08 ± 4.35	13.72 ± 5.19	27.73 ± 6.09
24	32.78 ± 3.4	24.48 ± 4.61	14.43 ± 5.75	21.64 ± 3.54	23.93 ± 7.08	35.06 ± 7.29
28	38.0 ± 3.75	26.06 ± 4.35	19.48 ± 4.32	24.61 ± 3.96	32.7 ± 7.83	35.18 ± 7.63

[a]Zero day is the day seeds were planted

TABLE IV

THE EFFECTS OF TEMPERATURE ON THE LEAKAGE OF ELECTROLYTES FROM SEVEN PLANT SPECIES GROWN AT 20°C OR 31°C

Plant Treated	Growth Temp. °C	Temperature of Slope Change In Conductivity Curve	Equation of the line $y=a+bx$	Confidence Level % $H_0: b_1 \neq b_2$	Mean of "t"	Standard Deviation of "t"	$(\bar{t}_{31°} - \bar{t}_{20°})$
Cabbage (*Brassica oleraceae*, c.v. Early Flat Dutch)	20	33	$y_1=3.248 + 0.155x_1$ $y_2=2.648 + 0.145x_2$	99	36	±4.24	8.5
		39	$x_1=2.189 + 0.123x_1$ $y_2=1.607 + 0.138x_2$	99			
Cabbage	31	44	$y_1=3.108 + 0.122x_1$ $y_2=1.908 + 0.153x_2$	99	44.5	±0.71	
		45	$y_1=0.667 + 0.1997x_1$ $y_2=1.228 + 0.245x_1$	99			
Corn (*Zea mays*, c.v. I. O. Chief)	20	47	$y_1=2.497 + 0.151x_1$ $y_2=2.531 + 0.250x_2$	99	46.3	±1.15	5.7
		47	$y_1=2.253 + 0.153x_1$ $y_2=1.130 + 0.178x_2$	99			
		45	$y_1=2.658 + 0.150x_1$ $y_2=0.901 + 0.183x_2$	99			
Corn	31	50	$y_1=2.828 + 0.100x_1$ $y_2=1.611 + 0.125x_2$	99	52	±3.46	
		56	$y_1=3.822 + 0.082x_1$ $y_2=0.724 + 0.141x_2$	95			
		50	$y_1=1.947 + 0.145x_1$ $y_2=-1.385 + 0.211x_2$	99			
Milo (Sorghum bicolor, c.v. Funk's hybrid 552)	20	43	$y_1=1.745 + 0.154x_1$ $y_2=0.484 + 0.182x_2$	99	43		5.3
		43	$y_1=4.322 + 5.405x_1$ $y_2=1.279 + 0.137x_2$	99			
		43	$y_1=0.243 + 0.206x_1$ $y_2=-1.357 + 0.243x_2$	99			
Milo	31	52	$y_1=2.206 + 0.138x_1$ $y_2=8.291 + 0.337x_2$	99	48.3	±4.22	
		45	$y_1=3.898 + 0.227x_1$ $y_2=2.203 + 0.266x_2$	99			
		48	$y_1=2.938 + 0.117x_1$ $y_2=1.197 + 0.155x_2$	99			
Oats (*Avena sativa*, L.	20	40	$y_1=2.414 + 0.152x_1$ $y_2=1.532 + 0.175x_2$	99	39.5	±0.71	5.0
		39	$y_1=2.463 + 0.150x_1$ $y_2=2.225 + 0.158x_2$	99			
	31	45	$y_1=2.542 + 0.150x_1$ $y_2=0.444 + 0.197x_2$	99	44.5	±0.71	
		44	$y_1=3.203 + 0.129x_1$ $y_2=0.254 + 0.203x_2$	99			
Soybeans (*Glycine max*, c.v. Bryso. 9)	20	43	$y_1=2.01 + 0.155x_1$ $y_2=0.740 + 0.184x_2$	99	40	±3.00	5.3
		40	$y_1=2.125 + 0.148x_1$ $y_2=1.787 + 0.158x_2$	95			
		37	$y_1=2.587 + 0.143x_1$ $y_2=1.393 + 0.176x_2$	99			
	31	46	$y_1=4.376 + 0.042x_1$ $y_2=1.707 + 0.128x_2$	99	45.3	±0.54	
		45	$y_1=2.180 + 0.151x_1$ $y_2=-0.212 + 0.204x_2$	99			
		45	$y_1=2.404 + 0.530x_1$ $y_2=1.447 + 0.175x_2$	99			
Tomato (*Lycopersicon esculentum*, L. c.v. Campbell's #17)	20	36	$y_1=2.509 + 0.142x_1$ $y_2=2.366 + 0.147x_2$	95	43	±9.4	8.0
		50	$y_1=2.247 + 0.132x_1$ $y_2=1.234 + 0.153x_2$	99			
	31	51	$y_1=2.077 + 0.147x_1$ $y_2=0.432 + 0.170x_2$	99	51		
		51	$y_1=2.406 + 0.104x_1$ $y_2=1.119 + 0.129x_2$	99			
Wheat (*Triticum aestivum*, L. c.v. Arthur)	20	41	$y_1=1.948 + 0.150x_1$ $y_2=-.569 + 0.210x_2$	99	40.1	±1.2	3.5
		39.3	$y_1=2.517 + 0.144x_1$ $y_2=1.851 + 0.160x_2$	99			
	31	42.5	$y_1=2.753 + 0.133x_1$ $y_2=1.565 + 0.161x_2$	99	43.5	±0.87	
		44	$y_1=2.815 + 0.124x_1$ $y_2=-0.016 + 0.187x_2$	99			
		44	$y_1=2.521 + 0.151x_1$ $y_2=1.118 + 0.183x_2$	99			

t = the apparent temperature at which electrolyte leakage occurred in a single replicate of that sample as shown by a change in slope of the conductivity curve.

$\bar{t}$ = the mean of the observed "t" values at a given growth temperature.

H_0 = hypothesis.

$b_1 ; b_2$ = slope of lines (see Figure 6).

$b_1 \neq b_2$ = slope of line is not equal to the slope of line 2.

Figure 2. The Growth Patterns of Wheat Grown at $20^{o}C$ (O) or $31^{o}C$ (□)

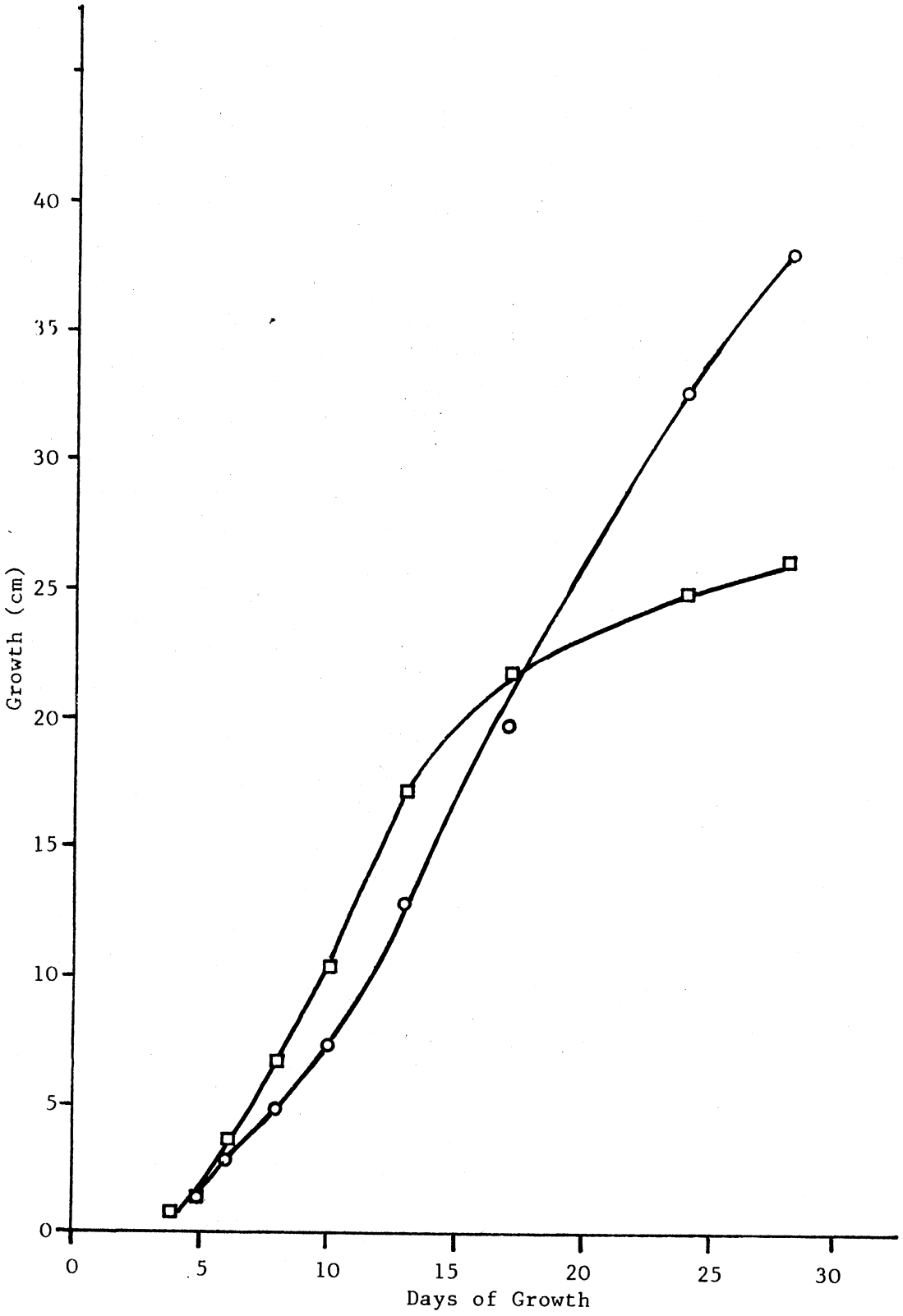

Growth (cm)
0
5
10
15
20
25
30
35
40
0
5
10
15
20
25
30
Days of Growth

Figure 3. The Growth Patterns of Corn Grown at 20°C (●) or 31°C (■)

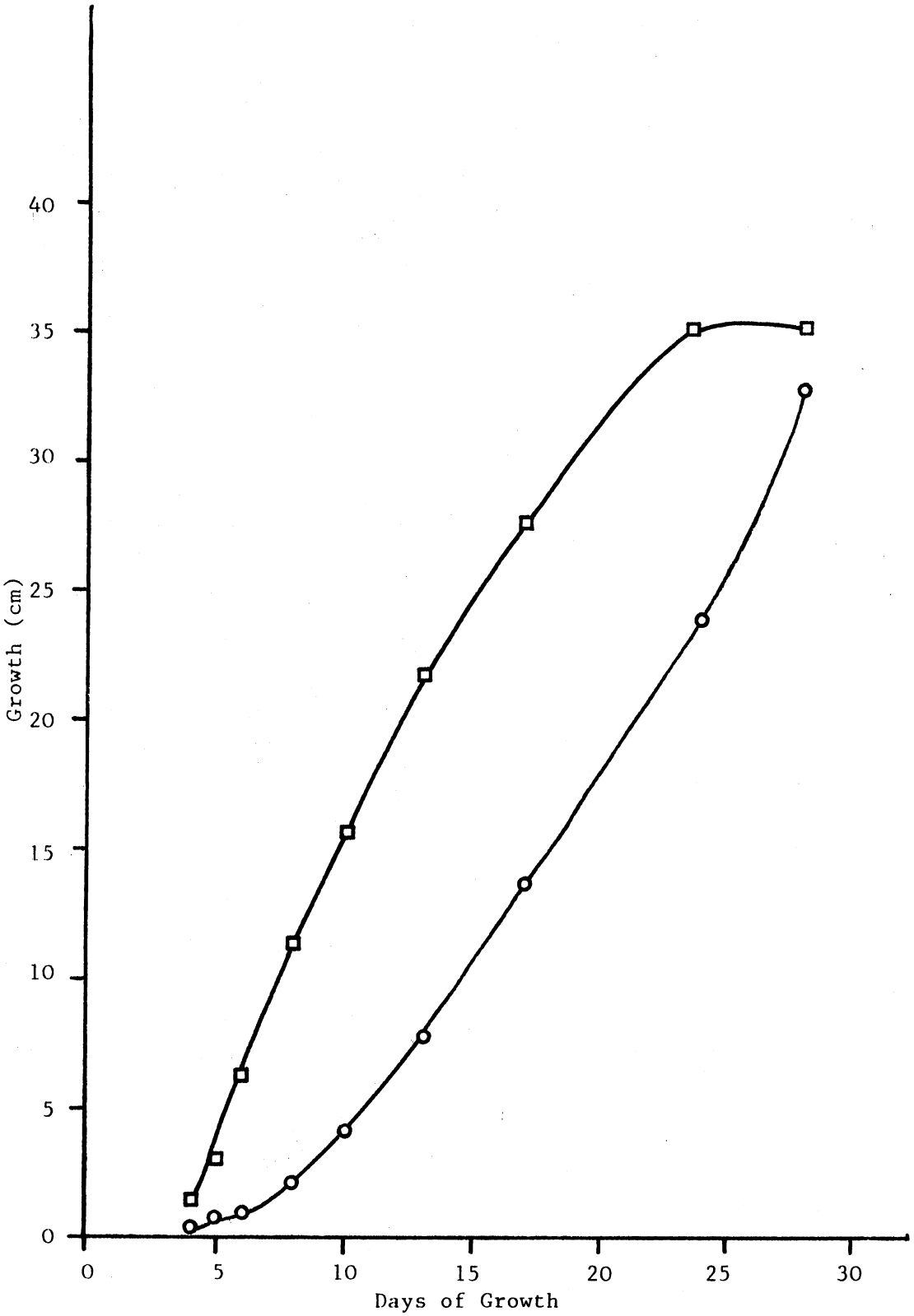

Growth (cm)
0
5
10
15
20
25
30
35
40
0
5
10
15
20
25
30
Days of Growth

Figure 4. The Growth Patterns of Milo Grown at 20°C (●) or 31°C (■)

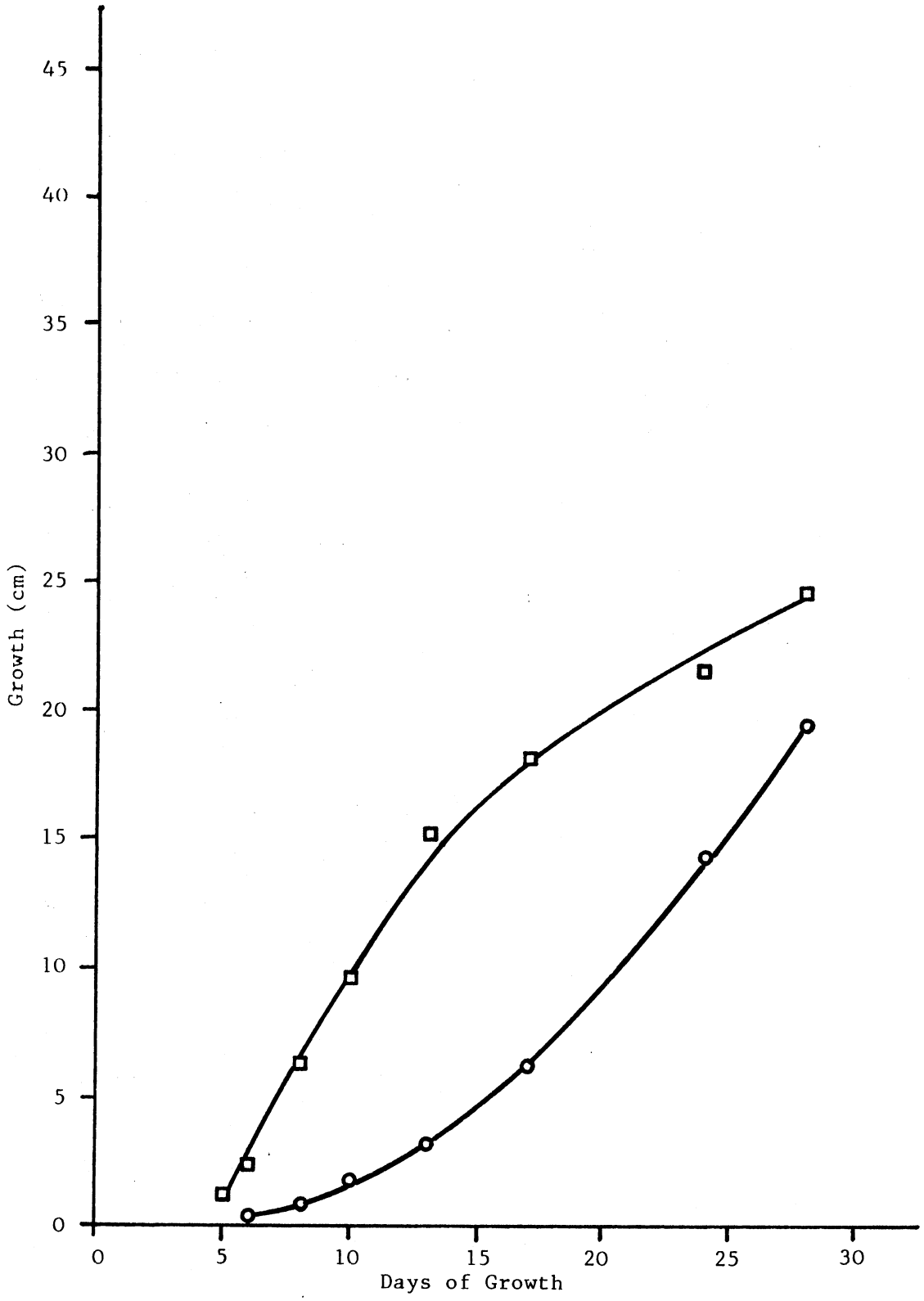

45
40
35
30
25
20
15
10
5
0
Growth (cm)
0
5
10
15
20
25
30
Days of Growth

Figure 5. Average Temperatures of Electrolyte Leakage From Isolated Chloroplasts From Seven Plant Species as Determined by Change in Slope of Conductivity Curve

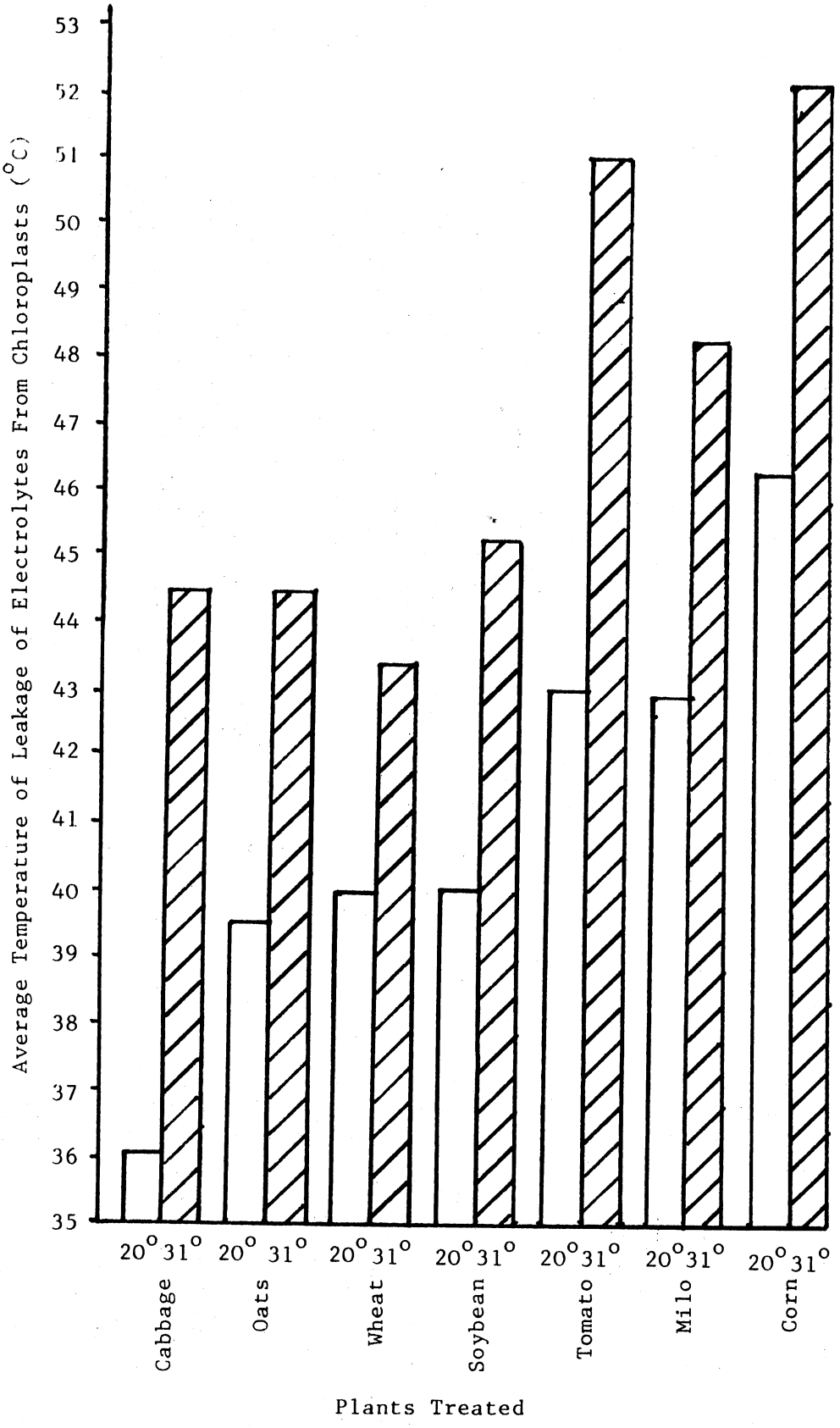
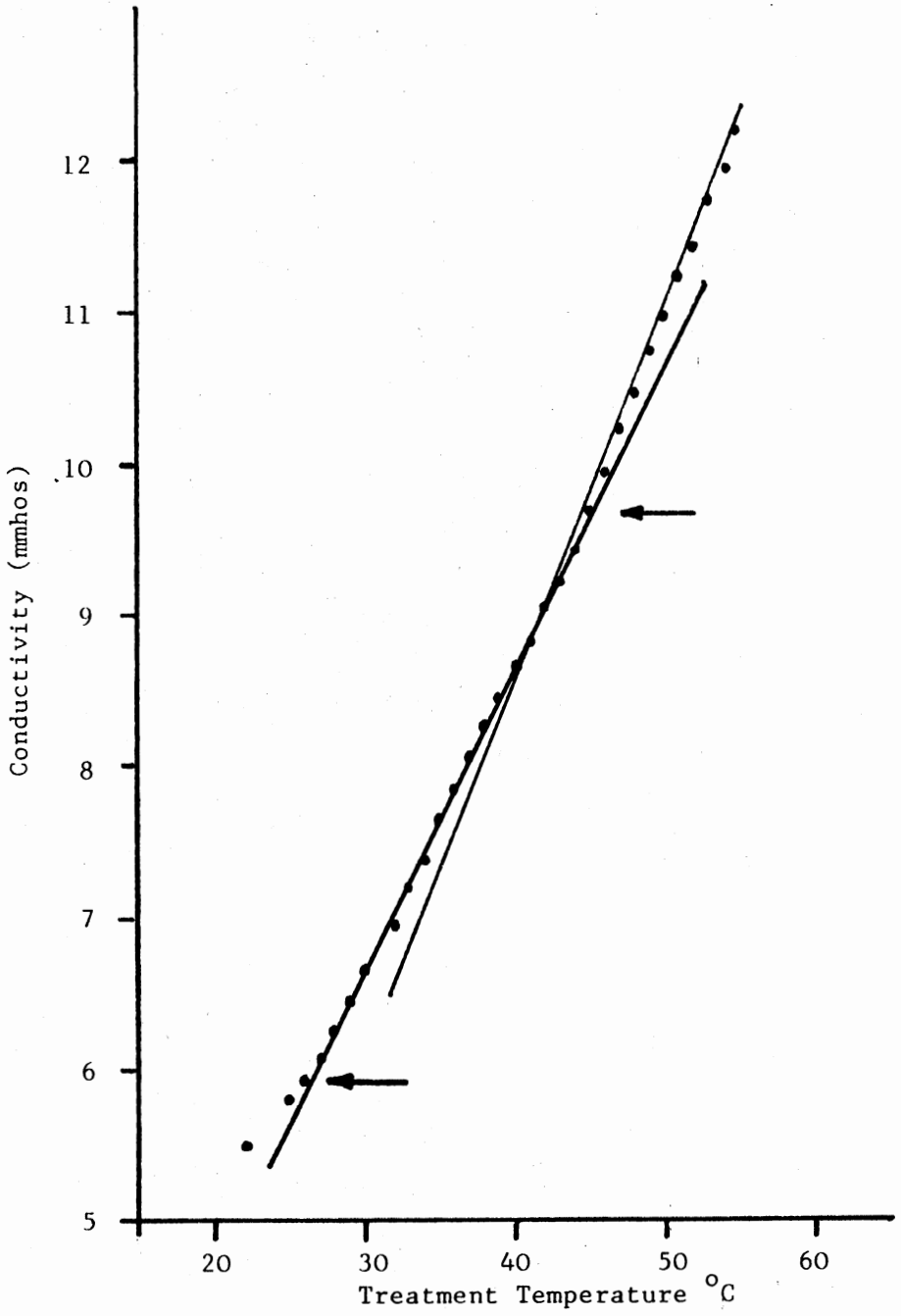

Average Temperature of Leakage of Electrolytes From Chloroplasts (°C)
53
52
51
50
49
48
47
46
45
44
43
42
41
40
39
38
37
36
35
20° 31°
Cabbage
Oats
Wheat
Soybean
Tomato
Milo
Corn
Plants Treated

TABLE V

THE EFFECTS OF TEMPERATURE ON MEMBRANE PERMEABILITY OF ISOLATED CHLOROPLASTS FROM CABBAGE GROWN AT 31°C AS DETERMINED BY CHANGES IN CONDUCTIVITY

Temperature °C	Conductivity mmhos	Temperature °C	Conductivity mmhos
22	5.5	41	8.82
25	5.8	42	9.09
26	5.94	43	9.26
27	6.08	44	9.41
28	6.26	*45	9.7
29	6.45	46	9.94
30	6.67	47	10.23
32	6.97	48	10.49
33	7.2	49	10.73
34	7.39	50	10.98
35	7.68	51	11.22
36	7.85	52	11.43
37	8.05	53	11.72
38	8.28	54.2	11.95
39	8.49	55	12.19
40	8.68		

Figure 6. The Effects of Temperature on the Membrane Permeability of Isolated Chloroplast From Cabbage Grown at 31°C as Determined by Changes in Conductivity. Points represent the actual data from Table V. Straight lines were calculated using the equations $y_1=0.6668 + 0.1997x_1$ for the lower line and $y_2=1.228 + 0.245x_2$ for the upper line. Arrows indicate changes in the slope of the conductivity curve.


Conductivity (mmhos)
12
11
10
9
8
7
6
5
20
30
40
50
60
Treatment Temperature °C

Cabbage, oats, soybeans and wheat seemed to be the heat sensitive plants among those studied. Cabbage chloroplasts (20°C) appeared to be the most heat sensitive with the leakage of electrolytes occurring at 36°C. The other three heat sensitive plants grown at 20°C appeared to leak electrolytes at approximately 40°C.

The heat sensitive plants grown at 31°C appeared to have comparable temperatures for electrolyte leakage from their chloroplasts ranging from 43.5°C in wheat to 45.3°C in soybeans, a difference of only 1.8°C.

Corn, milo, and tomato appeared to be significantly more heat tolerant than cabbage, oats, soybeans, and wheat. Electrolyte leakage from milo (43°C) and tomatoes (43°C) chloroplasts grown at 20°C occurred at temperature comparable to the leakage temperatures of the heat sensitive plants grown at 31°C.

The heat tolerant plants grown at 31°C appeared to have significant losses of electrolytes from their isolated chloroplasts at temperatures ranging from 48.3°C in milo to 52°C in corn with tomatoes intermediate at 51°C. However, the large standard deviations of corn at 31°C ($\pm$3.46°C) and milo at 31°C ($\pm$4.22°C) suggest that the apparent differences in heat sensitivity may be insignificant.

The differences in temperature for the leakage of electrolytes from chloroplasts isolated from plants grown at the two temperatures (Table IV) range from 3.5°C in wheat to 8.5°C in cabbage. Four of the seven plants analyzed had a temperature difference of around 5°C.

Graphical representation of the data for electrolyte leakage as determined by changing conductivity versus treatment temperature presented a problem as to the significance of the change in slopes at the

leakage temperature. Statistical analysis of the data using the t-test indicated that in all the graphs shown in this paper, the slopes observed above and below the temperature for leakage were significantly different at the 99% confidence level in thirty-two of the analyses and at the 95% confidence level in three.

Microscopic Evaluations of Heated Chloroplasts

Isolated wheat chloroplasts used in this experiment appeared normal at room temperature (22°C) (Figure 7). As the treatment temperature increased, chloroplasts exhibited swelling and an unexpected morphological condition I call "ballooning" (Figure 8--note arrow). The number of chloroplasts exhibiting swelling and "ballooning" increased with increasing temperature; ballooning occurred initially at temperatures well below that for leakage of electrolytes. Wheat grown at 31°C showed ballooning at 29°C (Table VI) while corn grown at 31°C showed ballooning at 27°C (Table VII). Ballooning continued to increase with increasing temperature (Figures 9, 10, 11 and Tables VI and VII) until fragmentation and clumping of chloroplasts (Figure 12) made it impossible to count ballooned chloroplasts. Clumping occurred in wheat (31°C) at 45°C–47°C accompanied by severe fragmentation. Similar treatment with corn chloroplasts (31°C) (Table VII) indicated some chloroplast fragmentation as low as 34°C but clumping was not observed even at 72°C.

Figure 7. Isolated Chloroplasts Treated at 22°C
Under Phase Microscopy (400x)

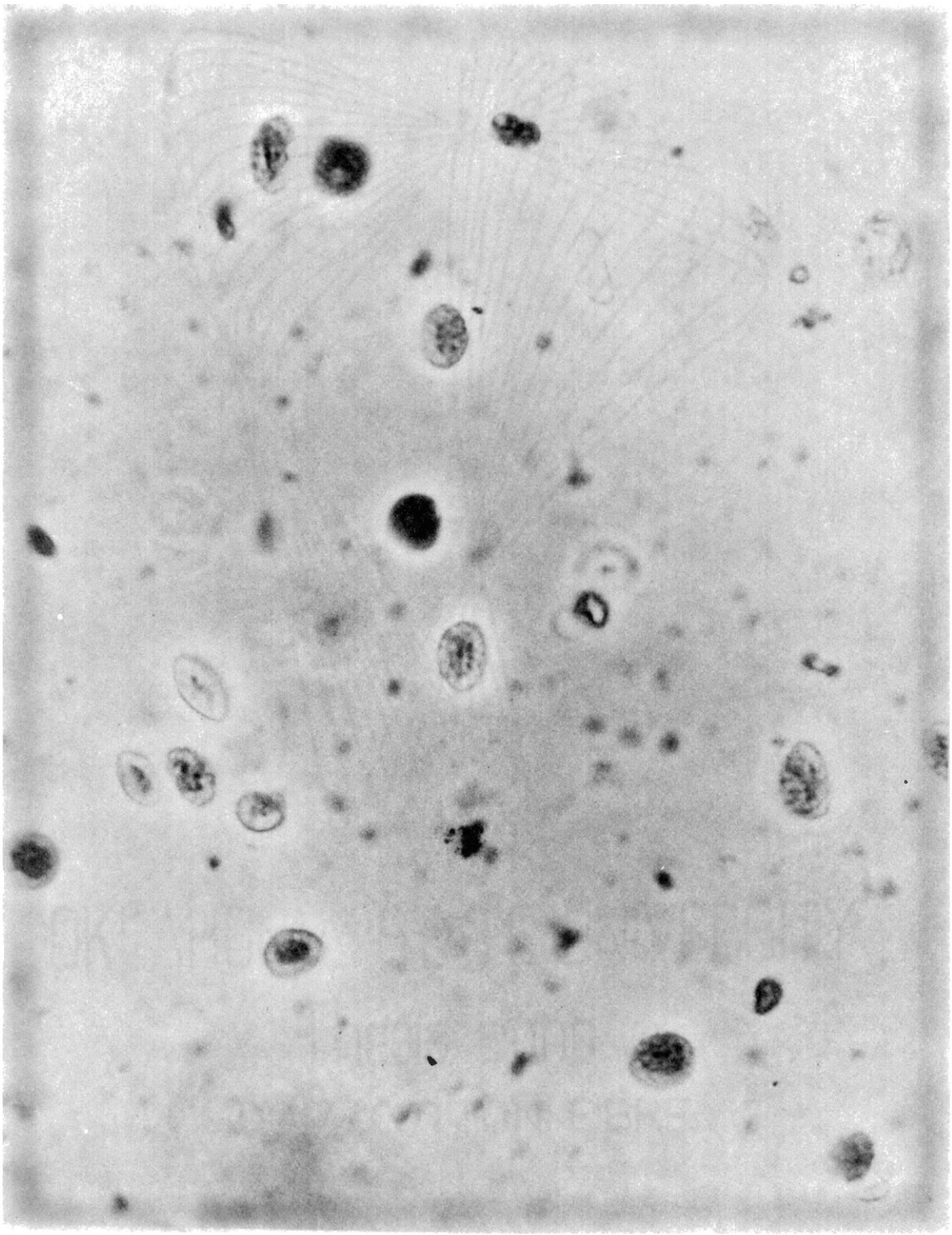


Figure 8. Isolated Chloroplasts Treated at 29°C
Under Phase Microscopy (400x)

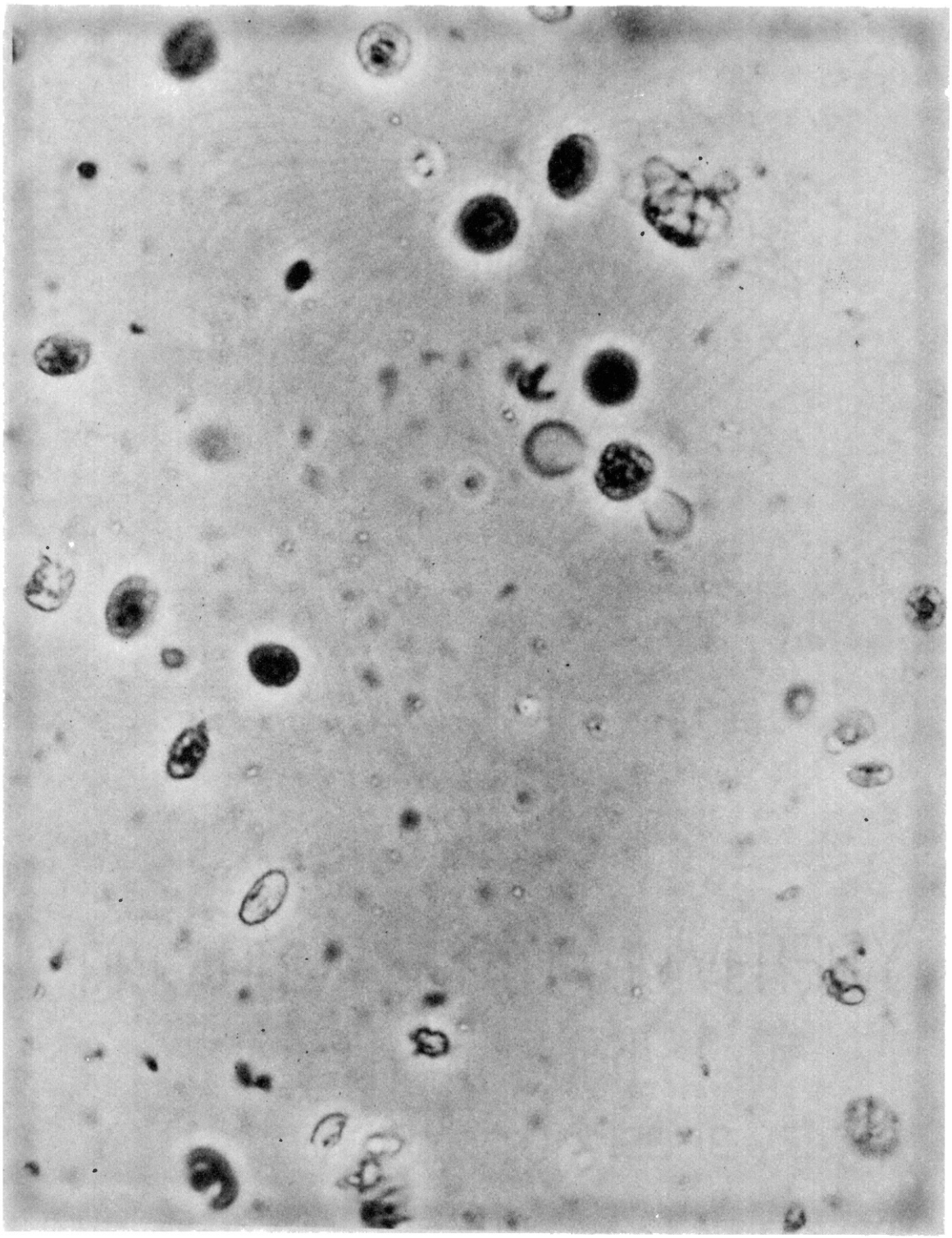


TABLE VI

THE EFFECTS OF TEMPERATURE ON THE CHLOROPLAST MEMBRANE AS DETERMINED BY THE "BALLOONING" PHENOMENON IN WHEAT (31°C)

Treatment Temperature (°C)	Number of Ballooned Chloroplasts per 100	Comments
22	0	
26	0	
29	4	
32	9	
36	22	
39	23	
42.5	36	Clumping and fragments.
45	--	Most chloroplasts have fragmented and clumping prevents counting ballooning.
48	--	Very few intact chloroplasts; extreme clumping and fragmentation

TABLE VII

THE EFFECTS OF TEMPERATURE TREATMENT ON CHLOROPLAST MEMBRANE INTEGRITY AS DETERMINED BY "BALLOONING"

Treatment Temperature	Number of Chloroplast Ballooning
22°C	0
27°C	11
29°C	31
32°C	48
34°C	43
37°C	51
40°C	73
43°C	64
46°C	59
49°C	79
52°C	72
56°C	72

Figure 9. Isolated Wheat Chloroplasts Treated at 32°C Under Phase Microscopy (400x)

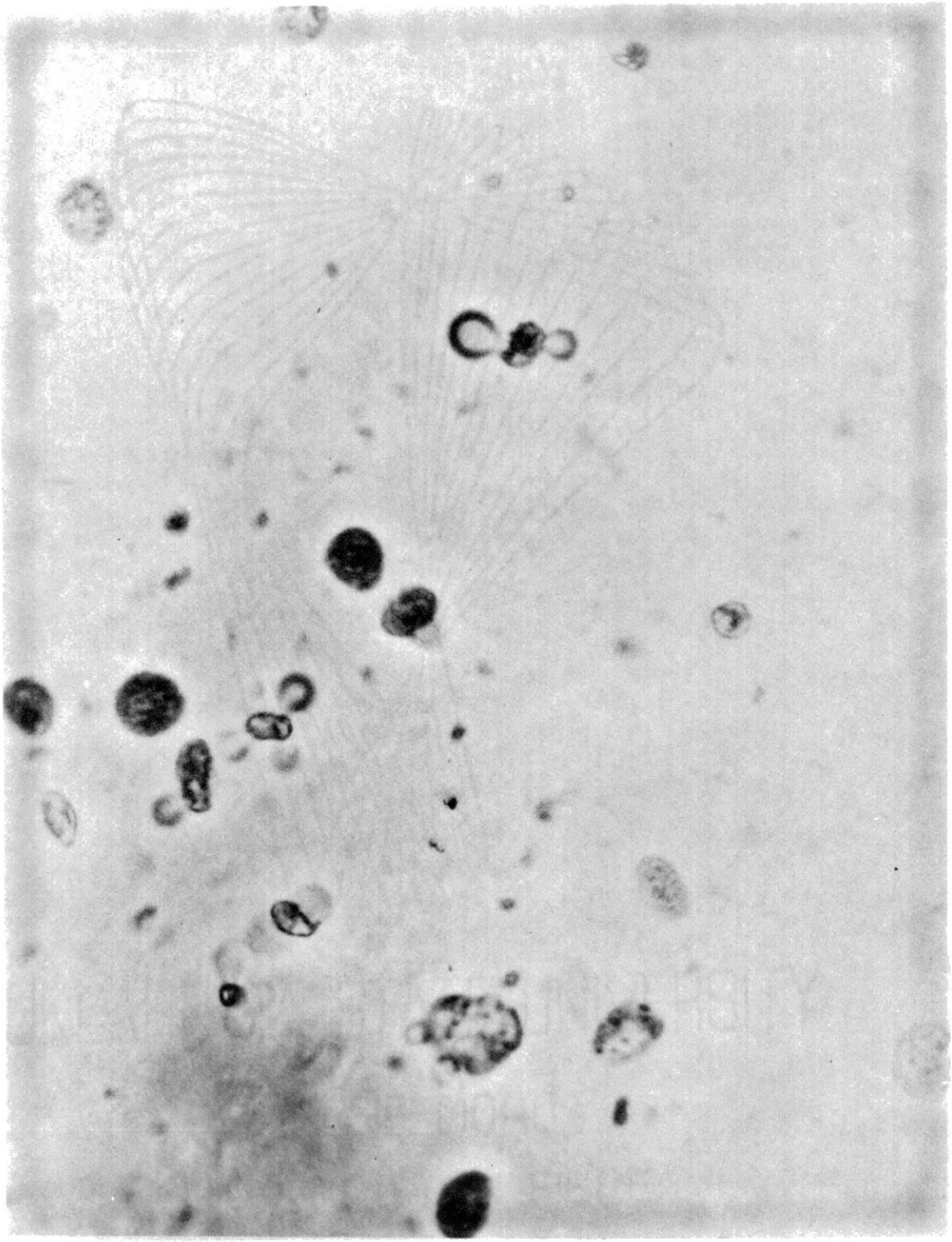


Figure 10. Isolated Wheat Chloroplasts Treated at 36°C Under Phase Microscopy (400x)

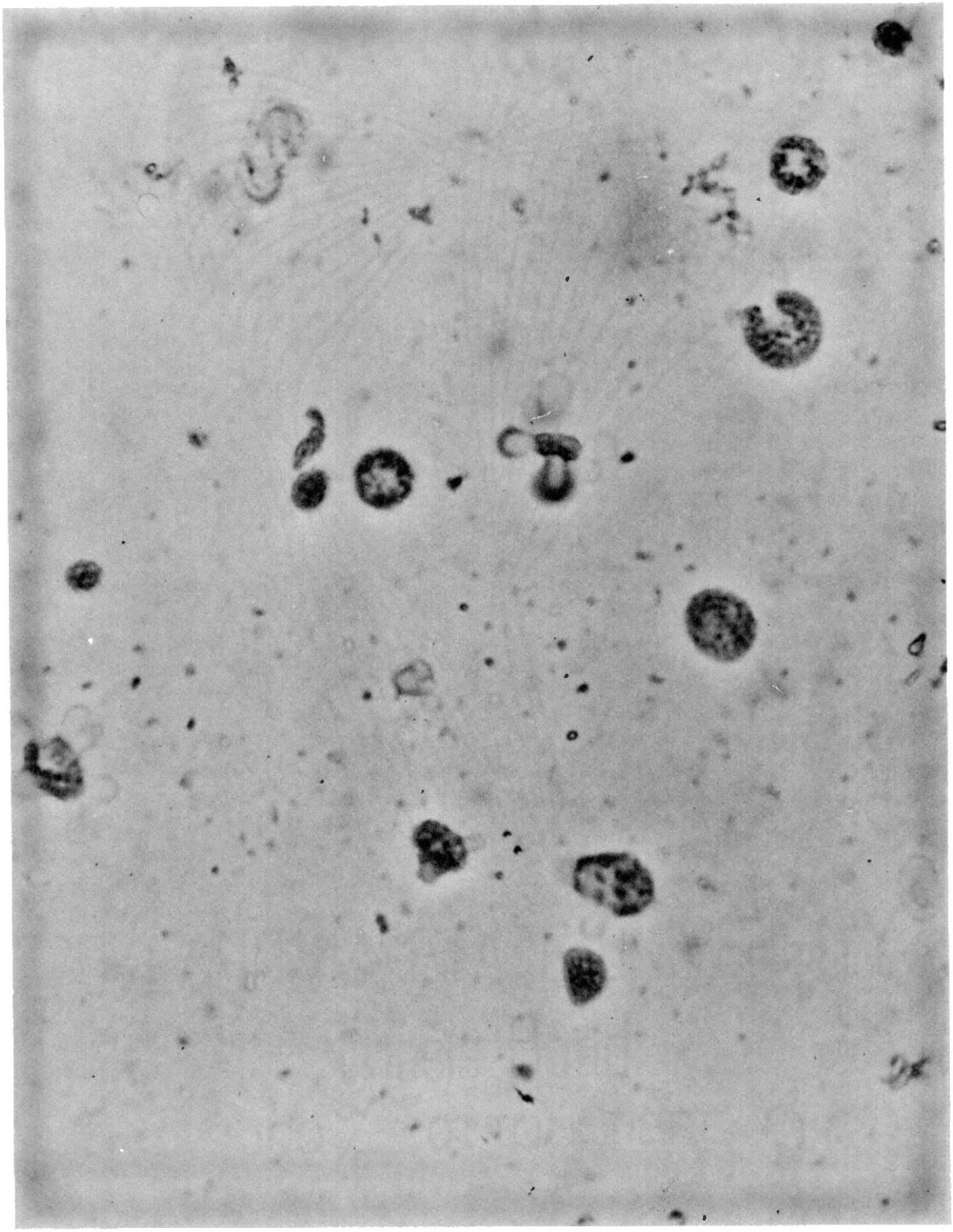


Figure 11. Isolated Wheat Chloroplasts Treated at 42°C Under Phase Microscopy (400x)

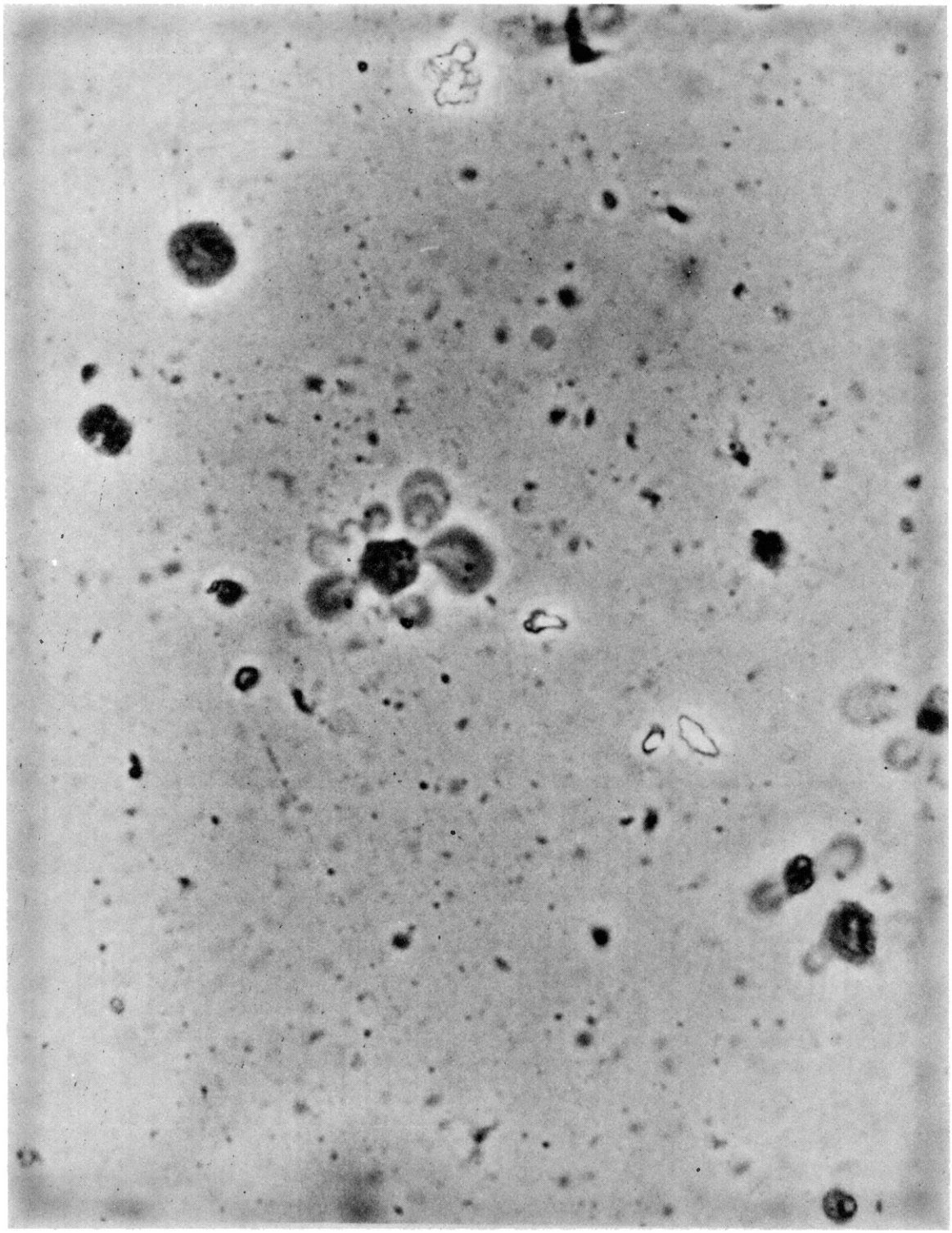
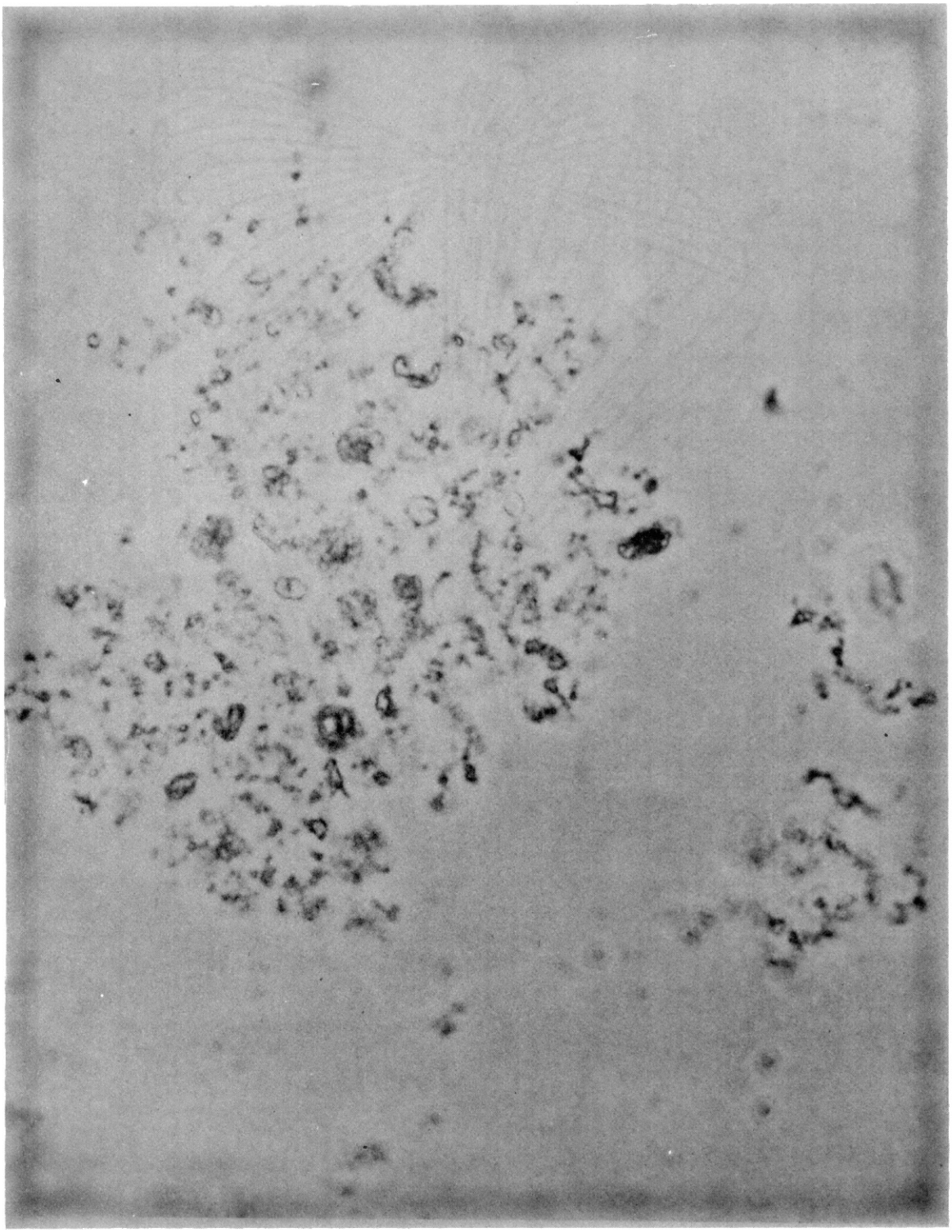


Figure 12. Isolated Wheat Chloroplasts Treated at 48°C Under Phase Microscopy (400x)



The Effects of Growth Temperature on the Fatty Acid Composition of Chloroplasts Isolated From Wheat

Palmitic acid increased in seven of nine lipids isolated when the growth temperature increased from 20°C to 31°C (Table VIII). The most significant increases occurred in phosphatidylglycerol (+21.3%) phosphatidylethanolamine (+20.3%), diphosphatidylglycerol (+16.9%), and phosphatidylserine (+13.9%). The smallest increase occurred in monogalactosyldiglyceride (+0.4%). Decreased levels of palmitic acid with increased growth temperature appeared in phosphatidyl choline (-0.9%) and sulfoquinovasyldiglyceride (-4.9%). Stearic acid (18:0) also increased at 31°C in seven of the nine lipids analyzed. The observed increases in stearic acid were much less than those observed in palmitic acid with the highest percentage increases occurring in phosphatidylglycerol (+7.5%). Other increases in stearic acid with increased growth temperature ranged from +0.4% to +3.3%, far below the observed changes in palmitic acid. Eicosaenoic acid (20:0) occurred in small amounts in all chloroplast lipids isolated from wheat grown at 20°C except phosphatidylserine and monogalactosyl diglycaride. Eicosaenoic acid (20:0) was present in trace amounts in sulfoquinosyldiglyceride and digalactosyldiglyceride and apparently absent from the other seven lipids isolated from chloroplasts grown at 31°C.

Higher ratios of saturated/unsaturated fatty acids occurred in seven of the nine chloroplast lipids isolated from wheat grown at 31°C compared to the chloroplast lipids isolated from wheat grown at 20°C (Table VIII). Major increases in the ratio of saturated/unsaturated

TABLE VIII

FATTY ACID DISTRIBUTION IN THE LIPID SPECIES
OF ISOLATED WHEAT CHLOROPLAST MOLE PERCENT

	16:0		16:1		16:3		18:0		18:1		18:2		δ 18:3		α 18:3		20:0		20:1		20:2		20:3	
	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C
phosphatidyl-serine	12.8	26.7	2.6	4.1	4.1	4.7	8.8	0.9	10.8	8.9	27.0	18.5	3.0	7.1	13.8	14.7	—[a]	—	—	—	10.7	8.3	6.2	6.0
phosphatidyl-inositol	8.7	13.0	1.7	4.5	9.2	5.2	8.3	9.0	8.6	10.2	29.8	25.9	0.3	3.8	11.9	14.1	1.7	—	—	—	14.8	10.2	5.1	5.1
phosphatidyl-choline	14.7	13.8	2.9	2.3	6.4	10.9	6.6	9.9	11.7	11.9	29.1	19.1	1.7	10.1	14.2	9.0	t[b]	—	—	—	9.4	13.1	3.2	t
sulfoquinovosyl-diglyceride	13.8	8.9	5.6	3.5	7.4	8.0	8.2	8.6	7.3	7.7	23.7	27.5	3.8	4.1	11.3	9.7	1.3	t	—	—	12.4	15.1	5.2	6.8
phosphatidyl-glycerol	4.1	25.4	1.2	3.3	4.4	3.4	5.6	13.1	6.6	8.8	29.9	15.8	3.3	4.9	16.9	10.3	2.6	—	—	—	19.6	7.0	5.6	7.0
phosphatidyl-ethanolamine	3.8	24.1	.7	4.7	8.0	5.3	4.5	7.2	9.0	8.9	27.6	18.2	3.6	3.8	16.3	11.8	t	—	—	—	20.4	9.4	6.2	6.5
digalactosyl-diglyceride	4.9	16.6	2.0	3.4	7.5	5.3	10.3	6.8	8.4	9.4	27.5	31.4	4.7	1.2	13.5	12.7	t	t	—	—	14.2	10.1	7.2	2.9
diphosphatyl-glycerol	5.4	22.3	2.3	16.0	7.0	3.0	6.1	9.3	8.3	8.1	34.1	23.5	1.7	3.6	15.0	13.3	4.5	—	—	—	13.7	5.2	2.0	5.8
monogalactosyl-diglyceride	12.3	12.7	8.4	6.8	4.2	6.2	8.0	10.6	6.5	8.6	33.9	22.0	1.1	3.9	12.3	10.0	—	—	—	—	8.7	9.4	4.5	9.9

[a] dashes indicate non-detectable quantities

[b] t indicates trace amounts

fatty acids occurred in phosophatidylglycerol (+40.7%) diphosphatidyldiglyceride (+27.2%) and phosphatidylethanolamine (+22.2%). The other lipids showed increased saturated to unsaturated fatty acid ratios ranging from +3.0% in phosphatidylcholine to +12.6% in digalactosyldiglyceride. Decreased saturated to unsaturated fatty acids levels in plants grown at 31°C occurred in sulfoquinovosyldiglyceride (-9.2%) and monogalactosyldiglyceride (-2.2%).

The two monenoic fatty acids found in wheat chloroplast lipids showed slight increases in concentration at the higher growth temperature (31°C)(Table IX). These fatty acids were relatively minor chloroplast lipid components. Oleic acid (18:1) was the most abundant monoenoic fatty acid while 11-eicosaenoic acid (20:1) was not present in any of the wheat chloroplast lipids analyzed. Oleic (18:1) increased slightly in six of nine wheat chloroplast lipids analyzed. These increases were small, ranging from +0.2% in phosophatidylcholine to +2.2% in phosphatidylglycerol. Decreases in oleic acid were also slight, ranging from -0.1% in phosphatidylethanolamine to the highest decrease of -1.9% in phosphatidylserine.

Palmitoleic (16:1) occurred in smaller quantities than oleic (18:1) in most wheat chloroplast lipids analyzed regardless of growth temperature. Like oleic, palmitoleic increased with increased temperatures in six of the nine chloroplast lipids isolated. The most significant increase occurred in diphosphatidylglycerol (+13.7%) with smaller increases in the other five lipids ranging from a low of 1.4% in digalactosyldiglyceride to +4.0% in phosphatidylethanolamine. Decreases in palmitoleic were also minor ranging from -0.6% in phosphatidylcholine to -2.1% in sulfoquinovosyldiglyceride.

TABLE IX

COMPARISON OF SATURATED TO UNSATURATED FATTY ACIDS, PERCENT DIENOIC ACIDS, PERCENT TRIENOIC ACIDS, AND THE SUM OF PERCENT DIENOIC AND TRIENOIC FATTY ACIDS IN LIPIDS ISOLATED FROM WHEAT GROWN AT 20°C OR 31°C

Lipid	(x 100) Sat/Unsat.		% Dienoic		% Trienoic		(Di + Tri) Polyunsat.	
	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C
Phosphatidylserine	27.6	38.1	37.7	26.8	27.1	32.5	64.8	59.3
Phosphatidylinositol	23.0	28.2	44.6	36.1	26.5	28.2	71.1	64.3
Phosphatidylcholine	27.1	31.1	38.5	32.2	25.5	30.0	64.0	62.2
Sulfoquinovosyldiglyceride	30.4	21.2	35.9	42.6	27.7	28.6	63.6	71.2
Phosphatidylglycerol	14.0	54.7	49.5	23.2	30.7	15.2	80.2	38.4
Phosphatidylethanolamine	9.1	31.3	48.0	27.8	34.1	27.4	82.1	55.2
Digalactosyldiglyceride	17.4	30.5	41.7	41.8	32.9	22.1	74.6	63.9
Diphosphatidylglycerol	19.0	46.2	47.8	28.7	25.7	25.7	73.5	54.4
Monogalactosyldiglyceride	25.5	23.3	42.6	31.4	22.1	30.0	64.7	61.4

Linoleic acid (18:2) was the most abundant fatty acid in wheat chloroplasts. Higher growth temperature (31°C) induced a lower concentration of linoleic acids in the wheat chloroplast lipids analyzed. Major decreases in 18:2 occurred at elevated growth temperatures in phosphatidylcholine (-14.1%), monogalactosyldiglyceride (-11.9%), diphosphatidylglycerol (-10.6%), phosphatidylcholine (-10.0%), phosphatidylethanolamine (-9.4%), and phosphatidylserine (-8.5%). A relatively minor decrease in 18:2 was recorded in only phosphatidylinositol (-3.9%). Linoleic acid increased with increased growth temperature in only sulfoquinovosyldiglyceride and digalactosyldiglyceride. Both apparently had relatively slight increases, +3.8% and +3.9%, respectively. However, these increases were more significant on the basis of total lipids since both sulfoquinovosyldiglyceride and digalactosyldiglyceride are two of the more abundant chloroplast lipids.

Hexadecatrienoic acid (16:3) was again found to be present in all wheat chloroplast lipids analyzed in quantities amounting to less than 10% in all lipids except phosphatidylcholine (-10.9% at 31°C). Hexadecatrienoic acid concentration did not vary with temperature in any predictable manner increasing with increased temperature in four lipids analyzed while decreasing in five. Major alterations in 16:3 concentrations at 31°C apparently occurred in phosphatidylcholine (+4.5%), phosphatidylinositol (-4.0%), and diphosphatidylglycerol (-4.0%).

Arachidic acid (20:0) was found to be present in measurable quantities in four of nine chloroplast lipids analyzed and in trace amounts in three others. Arachidic acid was most abundant in chloroplasts from wheat plants grown at 20°C. The highest concentration was 4.5% of the total lipid in diphosphatidylglycerol from chloroplasts grown

at 20°C and disappeared from chloroplasts grown at 31°C. Arachidic acid was found in trace amounts only in chloroplasts isolated from wheat leaves grown at 31°C. Chloroplasts showing trace amounts of 20:0 were sulfoquinovosydiglyceride and digalactosyldiglyceride, both very important chloroplast lipids.

α-linolenic acid was the most abundant trienoic fatty acid in isolated wheat chloroplasts. Increased growth temperature apparently induced decreases in α-18:3 in seven of nine chloroplast lipids analyzed. Major decreases appeared in phosphatidylglycerol (-6.6%) and phophatidylethanolamine (-4.5%). The only significant increase in α-18:3 occurred in phosphatidylinositol (+2.2%).

γ-linolenic acid was found in all wheat chloroplasts analyzed in quantities ranging from 0.3% to 10.1% of the lipids analyzed. γ-linolenic acid was found to be most abundant in phosphatidylcholine isolated from wheat chloroplast grown at 31°C, representing an increase of +8.4% over the amount present at 20°C. Surprisingly, the concentration of γ-18.3 increased in chloroplast lipids from wheat grown at 31°C in eight of nine lipids analyzed. Increases ranged from +8.4% in phosphatidylcholine to +0.2% in phosphatidylethanolamine. The only lipid showing decreased γ-18:3 at 31°C was digalactosyldiglyceride (-3.5%). Perusal of the data indicates γ-18:3 as a possible minor constituent of chloroplast lipids comprising less than 5% of most lipids analyzed.

11-14-Eicosadienoic acid (20:2) was found in all wheat chloroplast lipids analyzed and represented a significant proportion of the total lipid fatty acid. 11-14-Eicosadienoic acid was most abundant in phosphatidylglycerol isolated from wheat chloroplasts grown at 20°C representing 19.6% of the total fatty acids of this lipid, dropping to 7.0%

in chloroplasts grown at 20°C, a decrease of -12.6%. Other decreases in 20:2 at 31°C ranged from -2.1% in digalactosyldiglyceride to -11.0% in phosphatidylethanolamine. Increases in 20:2 at 31°C were comparatively small, ranging from +0.7% in monogalactosyldiglyceride to +3.7% in phosphatidylcholine.

11,14,17-Eicosatrienoic acid (20:3) represented a significant component of wheat chloroplast lipids in all lipids analyzed except phosphatidylcholine grown at 31°C where it occurred in trace amounts. 11,14,17-Eicosatrienoic acid decreased at 31°C in only three of the nine lipids analyzed with significant decreases occurring in only phosphatidylcholine (-3.2%) and digalactosyldiglyceride (-4.3%). Phosphatidylserine decreased only 0.2% while phosphatidylinositol maintained a constant level at the two growth temperatures. The surprising increases in 20:3 at 31°C ranged from +0.3% in phosphatidylethanolamine to +5.4% in monogalactosyldiglyceride.

Monenoic acids isolated from wheat chloroplast lipids did not appear to be significant markers for temperature induced changes. Therefore, the elimination of temperature induced changes in the degree of unsaturation of chloroplast lipids were based only on the dienoic and trienoic acids.

Dienoic fatty acids (18:2; 20:2) decreased with increasing temperature (Table IX) in all lipids analyzed except sulfoquinovosyldiglyceride (+6.7%) and digalactosyldiglyceride (+0.1%). Decreases in dienoic acid at 31°C ranged from -6.3% in phosphatidylcholine to -26.3% in phosphatidylglycerol. It is interesting to note that of the two galactolipids, monogalactosyldiglyceride (the most abundant chloroplast lipid) showed significantly decreased dienoic acid content at

31°C (-11.2%) while digalactosyldiglycerides showed an increase of +0.1% at this temperature.

Trienoic acids were the most unsaturated fatty acid components of chloroplast lipids (Table IX). Perhaps the most surprising aspect of the observed changes in chloroplast lipid fatty acids was the increase in trienoic acids in five of the nine lipids at 31°C.

These increases were generally small, ranging from +0.9% sulfoquinevosyldiglyceride to +7.9% in monogalactosyldiglyceride. The percent trienoic acid remained constant at the two growth temperatures in diphosphatidylglycerol (25.7%). Decreases in trienoic acids at higher growth temperature (31°C) ranged from -6.7% in phosphatidylethanolamine to -15.9% in phosphatidylglycerol.

The sum of trienoic and dienoic acids (Table IX) in isolated wheat chloroplast lipids showed decreased levels of unsaturation with increased temperature in eight of the nine lipids analyzed. Major decreases in dienoic and trienoic acid of chloroplasts grown at 31°C occurred in phosphatidylglycerol (-41.8%), phosphatidylethanolamine (-26.9%) and diphosphosphatidylglycerol (-21.1%). The only increase in polyunsaturation at 31°C occurred in sulfoquinovosyldiglyceride (+7.6%).

Analysis of the redistribution of lipid fatty acids with temperature suggests that increasing the growth temperature induces a shift toward fatty acids of smaller carbon number. This shift is significant even without regard to the degree of unsaturation of each fatty acid. The sixteen carbon fatty acids increased at the higher temperature while both the eighteen and twenty carbon fatty acids decreased at the higher growth temperature.

The Effects of Growth Temperature on the Fatty Acid Composition of Chloroplasts Isolated From Milo

The fatty acid composition of isolated milo (Funk's hybrid 522) chloroplasts was shown to be significantly affected by growth temperature (Table X).

Isolated chloroplasts from milo plants grown at 31°C were shown to have higher ratios of saturated to unsaturated fatty acids than those grown at 20°C (Table XI). All lipids identified except phosphatidylethanolamine and digalactosyldiglyceride were found to have higher ratios of saturated to unsaturated fatty acids. Major increases in saturated/unsaturated ratios occurred in phosphatidylserine (+20.9%), sulfoquinovosyldiglyceride (+20.6%) and diphosphatidylglycerol (+25.4%). These increased saturated/unsaturated ratios agree with previous data.

The major saturated fatty acid of chloroplast lipids was palmitic acid (16:0) except in phosphatidylcholine where stearate was most abundant. As expected, palmitic acid showed the greatest change in response to increased growth temperature. Stearic acid (18:0) changed with temperature but was generally unpredictable, increasing in some lipids and decreasing in others at higher temperature. Arachidic acid (20:0) occurred only in trace amounts in milo chloroplast. Such small concentration did not permit any temperature related comparisons.

Contrary to reports by Pearcy (1978) and others, hexadecatrienoic acid (16:3) was found to be a minor constituent of all lipids isolated. Plants grown at 20°C had slightly higher concentrations of 16:3 in seven of the nine lipids isolated from milo chloroplasts. Only phosphatidylserine (+1.5%) and sulfoquinovosyldiglyceride (+0.7%) showed higher

TABLE X

FATTY ACID DISTRIBUTION IN THE VARIOUS LIPID SPECIES OF MILO CHLOROPLASTS MOLE PERCENT

	16:0		16:1		16:3		18:0		18:1		18:2		18:3		18:3		20:0		20:1		20:2		20:3	
	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C
phosphatidyl-serine	14.4	19.6	1.0	6.0	5.9	7.4	6.5	12.5	8.9	4.7	28.2	18.8	4.3	3.5	13.9	12.3	t[a]	—[b]	—	—	11.7	10.6	5.3	4.7
phosphatidyl-inositol	14.8	16.1	5.5	8.3	6.6	4.6	9.2	8.9	6.7	6.7	25.1	24.6	2.6	2.1	13.1	11.6	t	t	t	—	11.4	10.4	5.0	6.8
phosphatidyl-choline	7.4	15.9	2.2	6.7	5.0	4.9	11.4	4.7	6.1	6.8	28.3	26.1	2.4	2.4	14.0	16.1	t	t	t	—	17.2	13.4	6.0	3.2
sulfoquinovosyl-diglyceride	9.6	24.0	6.8	3.2	6.6	7.3	6.6	4.5	10.7	7.9	27.9	16.5	4.1	4.1	14.1	10.8	t	t	—	—	11.9	16.6	8.2	5.2
phosphatidyl-glycerol	23.3	28.0	4.0	3.8	5.1	2.1	9.1	3.8	7.7	3.0	19.4	20.9	4.6	—	9.8	6.0	t	2.3	—	t	10.4	24.0	6.7	1.2
phosphatidyl-ethanolamine	15.0	15.9	4.7	6.4	5.8	2.2	9.4	6.2	7.3	6.5	20.0	28.4	4.7	1.0	9.2	10.0	1.5	—	—	—	12.2	19.7	10.2	3.6
digalactosyl-diglyceride	23.8	17.9	3.0	5.7	5.5	2.6	3.5	6.2	5.4	6.9	28.3	24.1	1.0	2.6	11.5	10.5	—	—	—	3.6	15.7	14.7	2.4	5.0
diphosphatyl-glycerol	19.2	27.9	3.9	5.3	8.3	3.0	6.5	9.6	7.5	9.2	22.1	15.4	3.2	5.4	11.7	11.8	t	—	—	—	12.1	7.2	5.4	5.2
monogalactosyl-diglyceride	13.0	15.9	4.4	4.0	5.0	4.4	6.6	8.2	9.3	8.3	27.2	28.8	2.5	—	13.8	14.8	—	t	—	t	12.6	12.5	5.6	3.5

[a] t indicates trace amounts

[b] dashes indicate non-detectable quantities

TABLE XI

COMPARISON OF SATURATED TO UNSATURATED FATTY ACIDS, PERCENT DIENOIC FATTY ACIDS, PERCENT TRIENOIC FATTY ACIDS, AND THE SUMS OF PERCENT DIENOIC AND TRIENOIC FATTY ACIDS IN LIPIDS ISOLATED FROM MILO GROWN AT 20°C OR 31°C

Lipid	(x 100) Sat/Unsat.		% Dienoic		% Trienoic		(Di + Tri) Polyunsat.	
	20°C	31°C	20°C	31°C	20°C	31°C	20°C	31°C
Phosphatidylserine	26.4	47.3	39.9	29.4	29.2	27.9	69.1	57.3
Phosphatidylinositol	32.0	33.0	37.0	35.0	22.3	25.1	59.3	60.1
Phosphatidylcholine	23.2	25.8	45.5	39.5	27.4	26.6	72.9	66.1
Sulfoquinovosyldiglyceride	19.3	39.9	39.8	33.1	32.9	27.4	72.7	60.5
Phosphatidylglycerol	47.4	51.7	29.8	44.9	26.2	9.3	56.0	54.2
Phosphatidylethanolamine	33.9	28.4	32.2	48.1	29.9	16.8	62.1	64.9
Digalactosyldiglyceride	36.1	31.8	44.0	38.8	20.4	20.7	64.4	59.5
Diphosphatidylglycerol	34.6	60.0	34.2	22.6	28.8	25.4	63.0	48.0
Monogalactosyldiglyceride	24.4	31.8	39.8	41.3	26.9	22.7	66.6	64.0

concentrations of 16:3 in chloroplasts from plants grown at 31°C.

Palmitoleic acid (16:1) increased in six of the nine lipids isolated from plants grown at 31°C. This data suggests not only an increased saturated/unsaturated at higher temperature but also a parallel decrease in carbon length as previously reported by Kleinschmidt and McMahon (1970). Sixteen carbon fatty acids represent larger percentages of total lipid fatty acids in chloroplasts isolated from plants grown at 31°C in seven out of nine lipids identified.

One of the more interesting aspects of these results was the apparent presence of γ-linolenic acid in all species of lipids isolated from milo chloroplasts. Gurr and James (1975) identified γ-linolenic as a minor component in animals and some algae and an important constituent of some plants. Although present in only small quantities (never more than 5.4%) it nevertheless showed slightly decreased levels at higher growth temperatures in five of the nine lipid species identified and remained constant in two lipids. Unexpected significant increases in γ-linolenic occurred at 31°C in diphosphatidylglycerol (+2.2%) and digalactosyldiglyceride (+1.6%) although the proportion of this fatty acid was still minor, representing 5.4% at its highest level in DPG.

Linoleic acid (18:2) shared with palmitic acid (16:0) the distinction as the most abundant fatty acid component of milo chloroplast lipids. Linoleic acid decreased at higher temperatures in six out of the nine lipids isolated. The most significant decreases occurred in phosphatidylserine (-9.4%) and sulfoquinovosyldiglyceride (-11.4%).

α-Linolenic acid has been reported by numerous investigators as the most abundant component of chloroplast lipids, especially the

galactolipids (Gardner, 1968; Allen et al., 1966; MacKender and Leech, 1974; Leese and Leech, 1976; Pearcy, 1978). These data did not confirm those reports. ∝-linolenic acid comprised a maximum of 16.1% of the total fatty acids of phosphatidylcholine and lesser amounts of other isolated lipid species. Also of interest was the apparent lack of significant, consistent temperature-induced changes in this lipid acyl residue. ∝-linolenic acid decreased at higher growth temperature ($31^{o}C$) in five out of nine milo lipids studied and remained constant in one.

Although ∝-linolenic acid (18:3) is generally the longest carbon length acyl residue of higher plant chloroplast lipids (Allen et al., 1966; Gardner, 1968; MacKender and Leech, 1974; Leese and Leech, 1976; Pearcy, 1978; Schwertner and Biale, 1973), one occasionally finds references to twenty carbon acyl residues. These twenty carbon fatty acids have been reported in algae (see Kates, 1970) and even in the leaves of ginkgo (Gellerman and Schlenk, 1972).

11-Eicosaenoic acid (20:1) was found only in trace amounts in five milo chloroplast lipids; only digalactosyldiglyceride ($31^{o}C$) had measurable quantities. 11,14-Eicosadienoic acid (20:2) was found in significant amounts in all lipids isolated from milo chloroplasts. However, only five out of the nine lipids had higher concentrations of 20:2 at $20^{o}C$ than at $31^{o}C$. 11,14,17-Eicosadienoic acid (20:3) was found in greater amounts in seven of nine lipids isolated. Although present primarily in the range of 5.6% of the total lipids, it does appear to respond to growth temperature, decreasing significantly with increasing growth temperatures in seven out of nine lipids analyzed. This is

not surprising considering the previous observation of increased saturated/unsaturated fatty acids at higher temperatures.

The degree of lipid unsaturation increased significantly at lower growth temperatures and decreased at higher temperatures. Only changes in dienoic and trienoic acids were considered important here. Dienoic fatty acids in milo chloroplasts decreased at higher temperatures in six of the nine isolated lipids. The major decreases occurred in phosphatidylglycerine (-10.4%) and phosphatidylglycerol (-11.6%). The other four lipids with reduced levels of dienoic acid at 31°C reflected changes of -2% to -6.7%. The three fatty acids which increased concentration of dienoic acid were phosphatidylglycerol (+15.1%), phosphatidylethanolamine (+18.2%), and monogalactosyldiglyceride (+1.5%). Both phospholipids showed significantly greater increases in dienoic acid content at 31°C than those which decreased at the higher growth temperature. Although monogalactosyldiglyceride did not increase as much as the two phospholipids PG and PE, the 1.5% increase in dienoic acids was important due to the extremely high content of MGDG in chloroplasts.

Milo plants grown at 31°C showed decreased concentrations of trienoic fatty acids in all lipids analyzed except phosphatidylinositol and digalactosyldiglyceride which increased only 2.8% and 0.3%, respectively. Major decreases appeared in phosphatidylglycerol (-16.9%) and phosphatidylethanolamine (-13.1%). The other lipids showed only small decreases in trienoic acid ranging from -0.8% to -5.5%.

When dienoic and trienoic were added together as a measure of changes in polyunsaturation in response to higher growth temperatures, results indicated decreased levels of polyunsaturated fatty acids in

all lipids analyzed except phosphatidylinositol and phosphatidylethanolamine, both relatively minor chloroplast lipids, which increased 0.8% and 2.8%, respectively, above the level determined from chloroplasts isolated from milo plants grown at 20°C. Major decreases in polyunsaturation at 31°C occurred in diphosphatidylglycerol (-1.5%) and sulfoquinosyldiglyceride (-12.2%) and phosphatidylglycerine (-11.8%).

Higher growth temperatures induced a redistribution of lipid fatty acids to shorter chain length. Sixteen carbon fatty acids increased with increased growth temperature while eighteen and twenty carbon fatty acids decreased.

The Lipid Composition of Wheat and Milo Chloroplasts

The mole percent of each lipid species, separated from the isolated chloroplasts of wheat and milo grown at 20°C and 31°C, was determined using the method of Allen and Good (1971). Data (Table XII) showed MGDG as the major lipid in chloroplasts of both plants at both growth temperatures with slightly smaller percentages of MGDG in milo chloroplasts at both temperatures than in wheat. MGDG was slightly higher at 31°C in wheat but higher at 20°C in milo.

DGDG was the second most abundant chloroplast lipid. DGDG represented a higher percentage of the total lipids in milo than in wheat at both growth temperatures. As was the case with MGDG, DGDG was higher at one growth temperature (20°C) in wheat but higher at the other growth temperature in milo (31°C), just the opposite of the relationship observed for MGDG.

No significant differences were apparent in the distribution of

TABLE XII

THE LIPID COMPOSITION OF WHEAT AND MILO CHLOROPLASTS GROWN AT 20°C OR 31°C
Mole Percentage of Total Lipids

Lipid	Wheat		Milo	
	20°C	31°C	20°C	31°C
Phosphatidylserine	1	1	1	1
Phosphatidylinositol	2	2	3	2
Phosphatidylcholine	6	5	6	7
Sulfoquinovosyldiglyceride	6	5	6	7
Phosphatidylglycerol	10	11	11	10
Phosphatidylethanolamine	1	1	1	1
Digalactosyldiglyceride	28	26	31	33
Diphosphatidylglycerol	1	1	1	1
Monogalactosyldiglyceride	41	44	40	38
Ratio of MGDG/DGDG	1.46	1.69	1.29	1.15

The results are the means of triplicate analyses.

the phospholipid in either species regardless of the growth temperatures.

The ratios of MGDG/DGDG were higher in wheat than milo at both growth temperatures. Calculations indicated MGDG/DGDG values of 1.46 at 20°C and 1.69 at 31°C in wheat chloroplasts and 1.29 at 20°C and 1.15 at 31°C in milo.

CHAPTER V

CONCLUSIONS

The effect of temperature on growth of an organism is very complex since virtually all cell processes within that organism are influenced simultaneously. Temperature effects may be, indeed should be, expected to act concomitantly with other environmental factors increasing the complexity of the temperature effects. Moreover, temperature-induced changes may cause altered patterns of development subsequent to the temperature change (Alexandrov, 1964).

Corn and milo grown at 31°C both grew faster during the early stages of growth than did plants grown at 20°C (Figures 3 and 4) agreeing with Langridge and Williams (1967). However, wheat (Figure 2) did not show this expected growth pattern. It is tempting to relate this altered growth pattern of wheat with the observed apparent higher thermal sensitivity of wheat as suggested by the leakage of ions.

The remarkable ability of thermophilic organisms to survive and grow at high temperatures may be attributed either to their exceptional capacity to rapidly synthesize and replace cellular constituents that are damaged or destroyed by heat or to the remarkable stability of their cellular materials at elevated temperatures. Raju et al. (1976) suggested that the lipids of biomembranes may play a significant role in thermophily. Fatty acid analyses of lipids extracted from thermophilic fungi suggest that saturated fatty acids predominate at higher temperatures.

The question still remains as to what factor(s) is(are) most important in conferring thermal stability.

Heat death in fresh water crayfish was shown to be accompanied by significant changes in Na^+ and K^+ concentrations which was attributed to a loss of integrity of membrane barriers separating intra- and extracellular compartments resulting in a loss of ionic gradient (Cossins, 1976).

Bowler et al. (1973) postulated that the membrane is the primary lesion of heat death and that changes in the properties of membranes during thermal acclimation may be responsible for thermal resistance. Bowler and Duncan (1967) presented evidence that both ($Na^+ + K^+$) activated ATPase and Mg^{+2} dependent ATPase are irreversibly inactivated by exposure to temperatures which cause death to the whole organism. However, Gladwell in 1975 (see Cossins, 1976) demonstrated that thermal inactivation of Mg^{+2} dependent ATPase is partially affected by thermal acclimation of the organism but not ($Na^+ + K^+$) activated ATPase.

Data reported by Cossins (1976) on the effects of high temperature on crayfish indicated that thermal acclimation has no effect on the overall phospholipid class distribution or content agreeing with data presented here. Further analyses of data led Cossins (1976) to conclude that the primary event leading to the breakdown of membrane permeability may not be a breakdown of the bulk membrane lipid bilayer due to increased fluidity or phase change but rather may be the result of changes in the specific lipid halos of specific membrane bound enzymes thus inactivating these enzymes.

Comparison of wheat and milo chloroplast lipids shows no significant temperature induced rearrangements. Data suggests that the major

alterations to chloroplast lipids involve the fatty acid building blocks.

The phase transition temperature in warm-blooded animals is well below body temperature and, with only a few exceptions, control of membrane fluidity is unnecessary. However, poikilothermic animals such as fish, crayfish, and others, do not have a constant body temperature. Therefore, the adaptation of the physicochemical properties of their membranes to changing temperatures may have considerable survival value. Data reporred by Farkas and Csengeri (1976), suggested that carp adjust their pattern of fatty acid synthesis to prevailing temperature. This response to temperature is both rapid and reversible. However, the nutritional state may have some effect on the intensity of the response.

Farkas and Csengeri (1976) suggested that differences in the fatty acid synthesizing systems would facilitate the production of the necessary chain lengths and saturated to unsaturated proportions at a particular temperature. They expressed confidence that this type of regulation is sensitive and rapid enough to ensure the proper functioning of cell membranes under changing conditions.

The well-known relationship between phase transition temperatures, carbon chain length, and double bonds explains why the same lipid species have different temperatures for phase transition. The proposal (Kimelberg and Papahadjopoulos, 1974) that the double kinks found in the Arrhenius plots of the enzyme halo lipids represent the lower phase transition from solid to a mixed liquid-crystalline state while the upper kink represents the transition to a completely fluid state is most intriguing. The application of this proposal to the leakage of

chloroplast electrolytes observed in this experiment as indicated by the kinks in the electrolyte leakage plots could enhance our understanding of these events. The suggestion that the leakage of sodium ions acts as an important indicator for the onset of fluidity changes (Kimelberg and Papahadjapolous, 1974) supports this possibility.

It is possible that leakage of electrolytes from the membrane may be due to the thermal destruction (denaturation) of the active transport mechanism rather than the membrane integrity. However, the temperature range of leakage does not support this concept.

It is tempting to postulate that lipid fatty acid rearrangement confers thermal stability by increasing the phase transition temperature due to the higher melting temperatures of more saturated lipid fatty acid. Unfortunately, there is no conclusive evidence extending the phase transition temperature to 35°C or 45°C where thermal inactivation of Atriplex lentiformis photosynthesis occurs (Pearcy, 1978). However, this writer is not convinced that the kinks in the conductivity plots observed in this paper do not suggest membrane lipid phase transitions, since kinks were also observed at the temperatures where Murata et al. (1976) and Kimelberg and Papahadjopolous (1974) reported phase changes (see Figure 6). However, these lower kinks occurred here at approximately 24°C-27°C no matter which plant was used or the growth termperature.

The fatty acid rearrangement associated with increased temperatures were similar to those reported in leaf senescence. Newman et al. (1973) found that senescence resulted in a decline in linolenic acid (18:3) with a concomitant relative increase in palmitic acid (16:0). Newman et al. (1973) further reported that sucrose delayed the

breakdown of fatty acids during senescence comparing surprisingly well with the protective effect of sucrose on thermal induced loss of membrane integrity of plants (Santarius, 1973).

Results of these experiments suggest the presence of 20 carbon acyl groups associated with all chloroplast lipid classes analyzed. This data is contrary to previous fatty acid analyses of chloroplasts isolated from corn (Leese and Leech, 1976), Atriplex lentiformis (Pearcy, 1978), spinach (Allen et al., 1966, and Allen and Good, 1971), barley (Newman et al., 1973), Vicia faba (MacKender and Leech, 1974), and others, where 18 carbon acids were the longest reported.

Unknown peaks among the 18 carbon fatty acids and beyond were identified with standards obtained from Sigma Chemical Company. The known peaks corresponded with the relative retention times of these standards (see Table I).

Twenty carbon fatty acids have been reported in numerous lower plants including algae (Jamieson and Reid, 1976), fungi (Sawicki and Pisano, 1977), and others. Twenty carbon fatty acids have also been isolated from poikilothermic animals, e.g., crayfish (Cossins, 1976) and carp (Farkas and Csengeri, 1976). Reports of twenty carbon fatty acids in Ginkgo biloba (Gallerman and Schlenk, 1972) leaves was of special interest because of its position as a primitive coniferophytae. If twenty carbon chain length fatty acids are not present in the leaves of higher plants, what would be the selective advantage of their removal?

Hitchcock and Nichols (1971) suggested that mineral deficiencies and low light intensity decrease the concentration of 18:3 which could

explain the unexpected low concentration of linolenic acid in these experiments.

Leese and Leech (1976) showed electron micrographs of isolated Zea mays chloroplasts with a small separation of the inner and outer chloroplast envelope membranes increasing with increasing maturity of developing maize leaves, but they made no mention of this phenomenon.

Heldt and Sauer (1971) described a similar phenomenon in isolated spinach chloroplasts as a separation between the outer and inner envelope membranes with a large empty space between. They concluded that the outer chloroplast envelope is unspecifically more permeable to sucrose and other molecules of similar size.

If this is true, results of the temperature-induced ballooning suggest that the outer envelope is more thermal sensitive than the inner envelope allowing the sucrose or mannitol to leak into the intermembrane space producing the balloon-like structures. Neither Heldt and Sauer (1971) nor Leese and Leech (1976) reported the dramatic ballooning observed in these experiments.

It is unfortunate that ballooning chloroplasts did not increase significantly in number at the temperature of apparent loss of membrane integrity as indicated by the conductivity data.

Chloroplasts with intact envelopes appear bright green with bright halos under the phase microscope. Chloroplasts lacking intact envelope membranes appear gray and lack the halos. All ballooned chloroplasts were bright green with halos suggesting that the envelopes were still intact.

Where does this extra membrane material forming the "balloon" originate? There are several possibilities:

(1) de novo synthesis of new membrane material;

(2) the extrusion of lamellae membranes through both the inner and outer envelopes which break and reform with the lamellar membranes without loss of continuity and integrity of the outer chloroplast membrane; or

(3) a stretching of the outer envelope membrane, possibly similar to that described by Johnson (1978), with an influx of the buffered sucrose solution into the inter-membrane compartment.

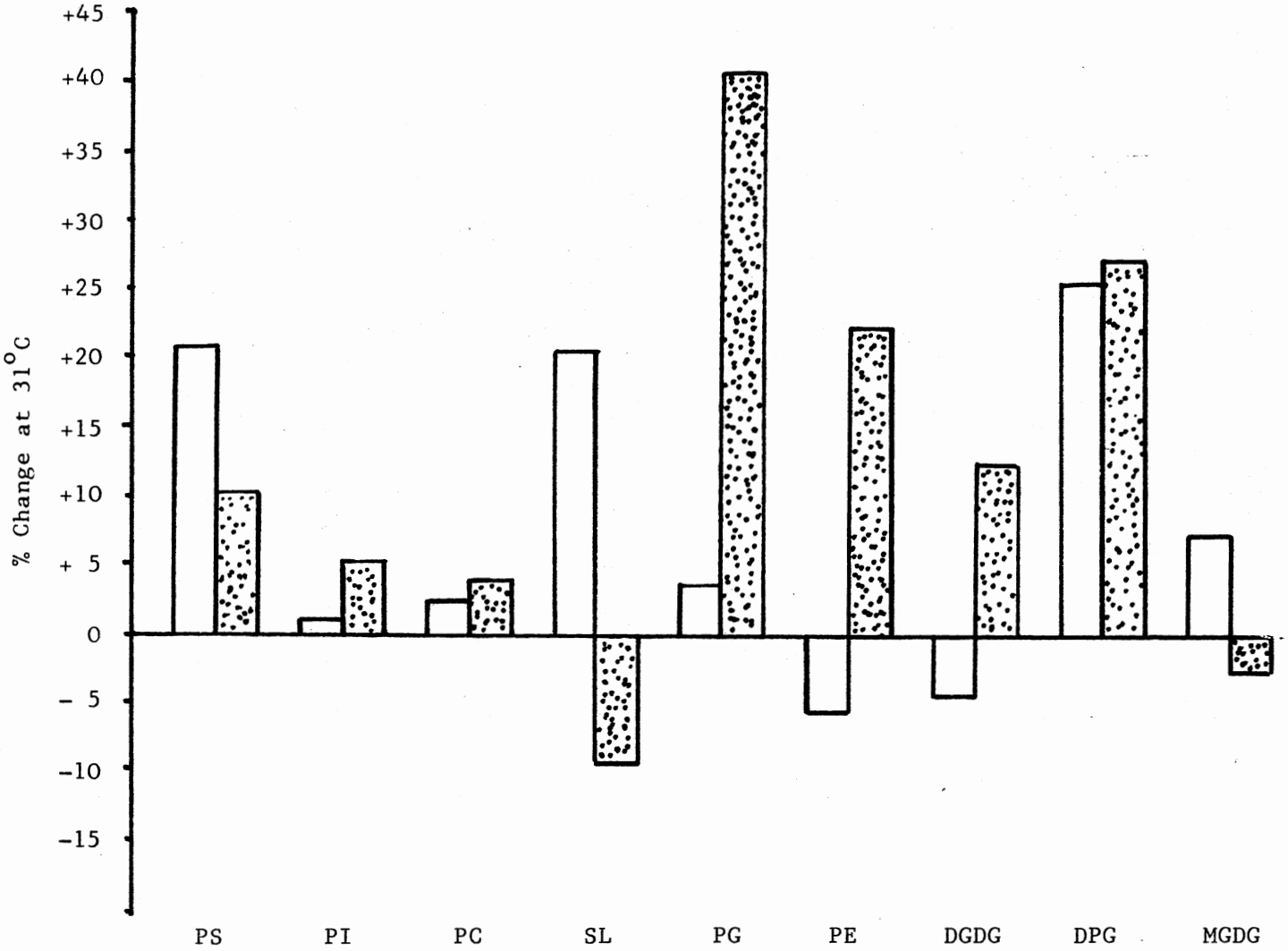
The evidence seems to support a stretching phenomenon, but this requires further study.

Another interesting question initiated by these observations is whether or not ballooning can be repaired. Lowering the temperature of the chloroplast suspension to room temperature (22^{o}C) had no observable effect (unpublished data).

Figure 13 supports the reported conclusions of Kleinschmidt and McMahon (1970), Holton et al. (1964), and others, indicating that plants adjust to higher growth temperatures by increasing the ratio of saturated to unsaturated fatty acids of their lipids. It is tempting to relate these changes to the relative thermal sensitivity of each plant species analyzed.

Wheat appears to adjust the saturated/unsaturated ratio (Figure 13) primarily in PG, DPG, PE and DGDG in decreasing order (PG highest) while milo adjusts primarily in OPG, SL, PS and MGDG. Since MGDG, DGDG, and PG are the most abundant chloroplast lipis, as shown in Table XII, one could assume that changes in these lipids would be most important in conferring thermal stability. Bjorkman et al. (1976) included SL with PG as the major lipids involved in conferring thermal

Figure 13. Comparison of the Changes in the Ratios of Saturated to Unsaturated Fatty Acids in Wheat and Milo Chloroplasts When the Growth Temperature is Increased From 20°C to 31°C. Wheat is shaded.


+45
+40
+35
+30
+25
+20
+15
+10
+ 5
0
- 5
-10
-15
% Change at 31°C
PS
PI
PC
SL
PG
PE
DGDG
DPG
MGDG

stability due to Anderson's (1975) theory identifying PG and SL as the membrane protein boundary lipids (halos) even though SL is a relatively minor chloroplast component (5-7%).

Membranes have been described according to the fluid mosaic model (Singer and Nicholson, 1972) as having a patchwork distribution of lipids rather than homogeneous distribution. Some lipids compose the fluid bilayer of the membranes while others form the boundary lipids of the membrane proteins (Jost et al., 1973; Dehlinger et al., 1974). Pearcy (1978) recognized the importance of Anderson's (1975) identification of galactolipids (MGDG and DGDG) as components of the membrane fluid bilayer and SL and PG as protein boundary lipids (halos).

These observations make two approaches for explaining the involvement of lipids in conferring thermal stability of membranes very attractive:

(1) maintenance of the necessary fluidity levels of the fluid bilayer, and

(2) maintenance of the membrane protein boundary lipid (halos) at the required fluidity level, especially those involving membrane bound enzymes.

Assuming that SL and PG are indeed the major components of the membrane protein lipid halos (Anderson, 1975), it can also be assumed that any alteration in these lipids that might affect the activity of membrane bound enzymes (Cossins, 1976) with possible concomitant effects on membrane permeability properties.

The difference in the ratio of saturated to unsaturated fatty acids from isolated wheat and milo chloroplasts grown at 20°C and those grown at 31°C showed the expected increases at higher

temperatures. However, all lipid species did not alter their lipid fatty acid equally. Chloroplast PG isolated from wheat grown at 31°C showed increased saturated/unsaturated ratio of more than 40% (Figure 13), while PG isolated from milo chloroplasts increased less than 4%. Analyses of SL fatty acid from wheat and milo showed increases of 20% in milo (31°C) and an unexpected decrease of 9% in wheat (31°C).

If, as the evidence suggests, milo is more thermal tolerant than wheat, then changes in saturated/unsaturated fatty acids of the two proposed membrane fluid bilayer lipids indicate that SL is more effective than PG in conferring thermal stability to these membranes.

Secondly, if the changes are compared that occur in dienoic fatty acids (Figure 14) in SL and PG at higher temperatures, it is found that the dienoic fatty acids decrease in wheat (-27%) PG while increasing in milo (+15%) PG. However, dienoic fatty acid increases in SL (+6%) in both wheat and milo. Analyses of trienoic fatty acids (Figure 15) shows comparable decreases in PG of both wheat and milo (-17% and -16%, respectively) while in SL, milo showed a decrease in trienoic acids (-6%) as would be expected. Wheat showed a slight increase (+1%) which could be insignificant. The sum of dienoic and trienoic chloroplast lipid fatty acids (Figure 16) showed a large decrease in wheat PG (-42%) at 31°C but a much smaller decrease in milo PG (-2%). Milo (31°C) showed a decrease in the sum of trienoic and dienoic fatty acids in SL (-13%) while wheat (31°C) registered a surprising increase (+7%).

Assuming that the data indicating that milo is more heat tolerant than wheat and that increased saturated/unsaturated values increase thermal stability, the data presented suggests:

Figure 14. Comparison of the Changes in the Percent Dienoic Fatty Acids in Wheat and Milo Chloroplast Lipids When the Growth Temperature is Increased from 20°C to 31°C. Wheat is shaded.

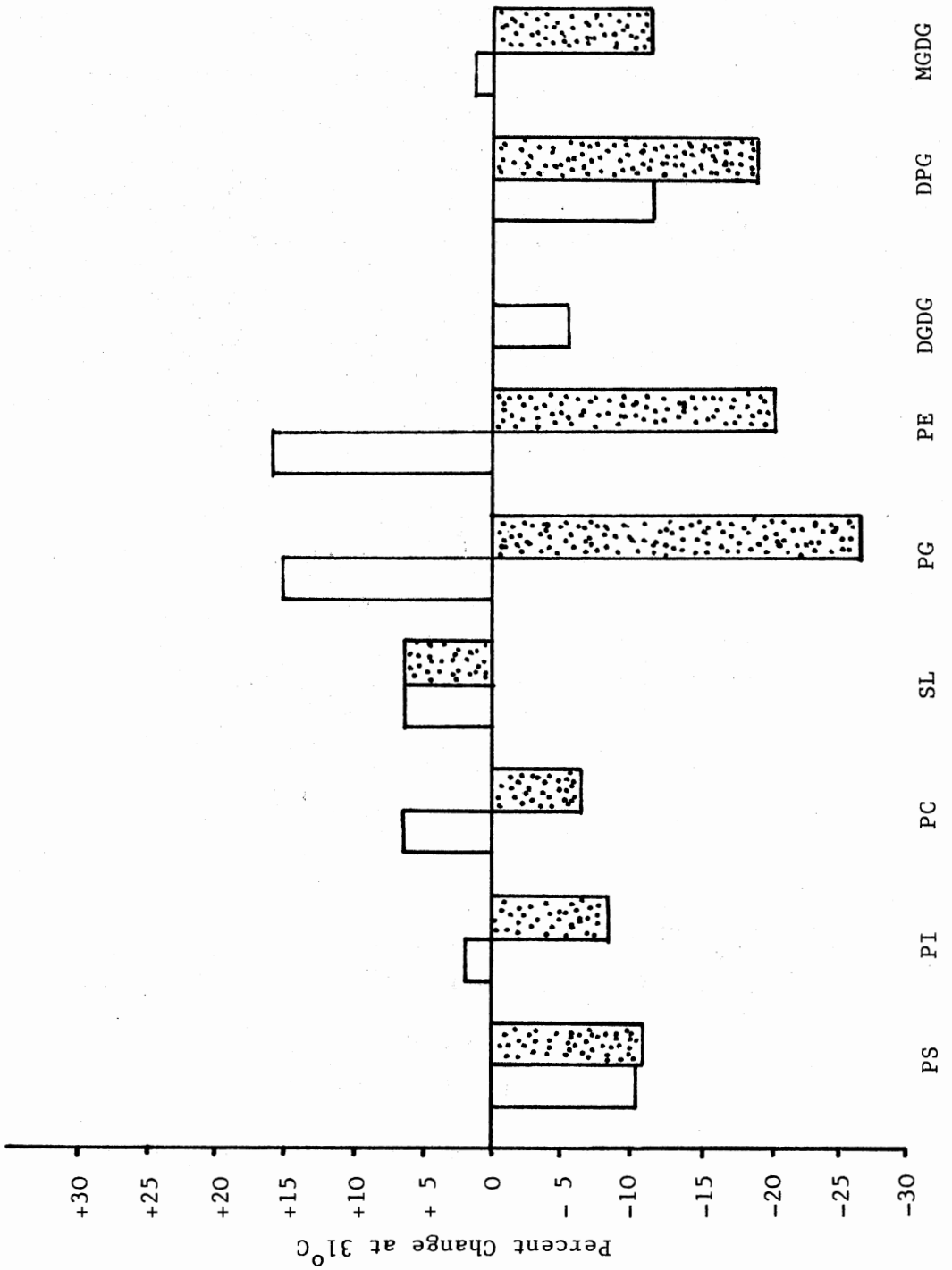

Percent Change at 31°C
+30
+25
+20
+15
+10
+5
0
-5
-10
-15
-20
-25
-30
PS
PI
PC
SL
PG
PE
DGDG
DPG
MGDG

Figure 15. Comparison of the Changes in the Percent Trienoic Fatty Acids in Wheat and Milo Chloroplast Lipids When the Growth Temperature is Increased From 20°C to 31°C. Wheat is shaded.

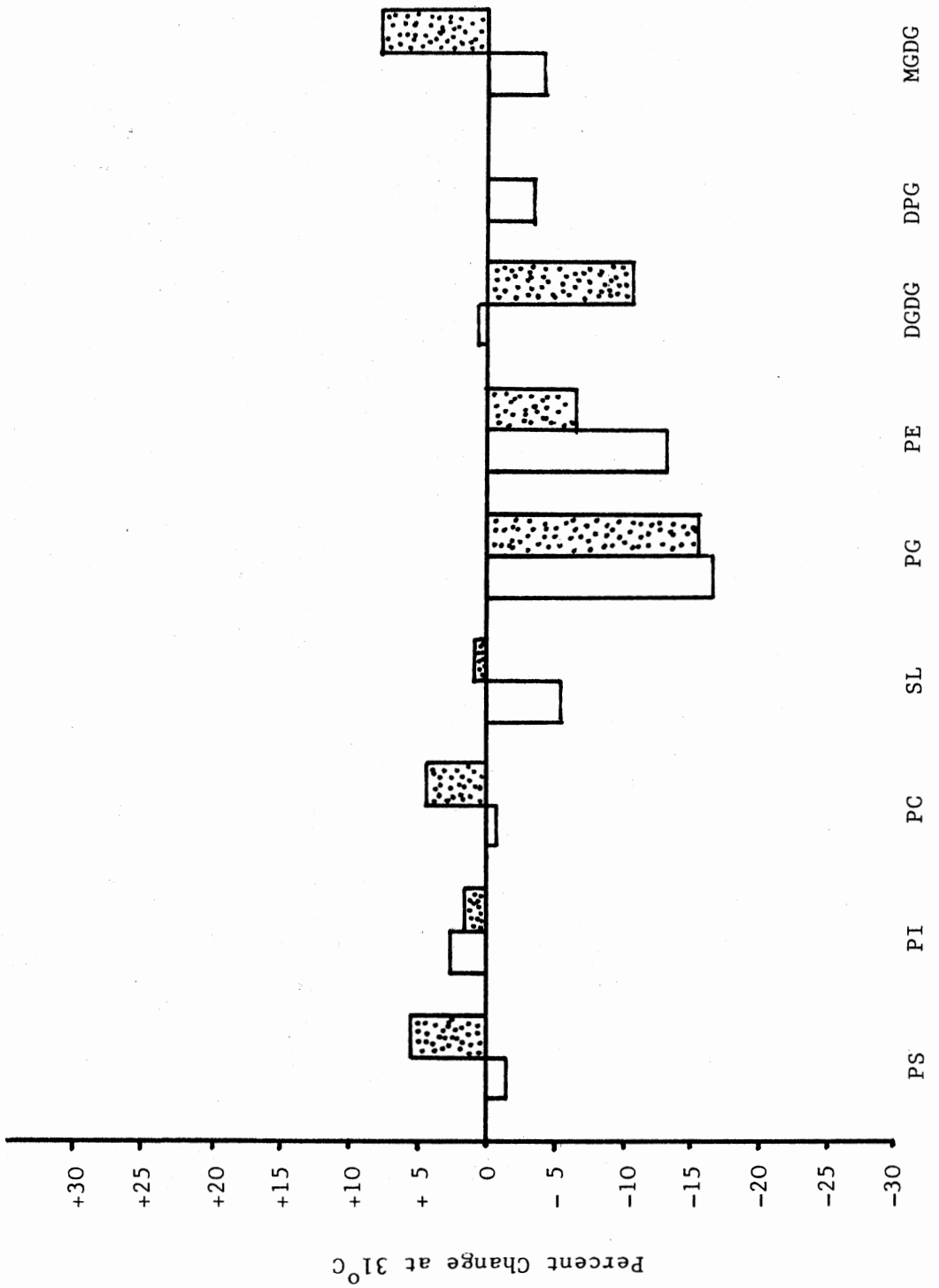
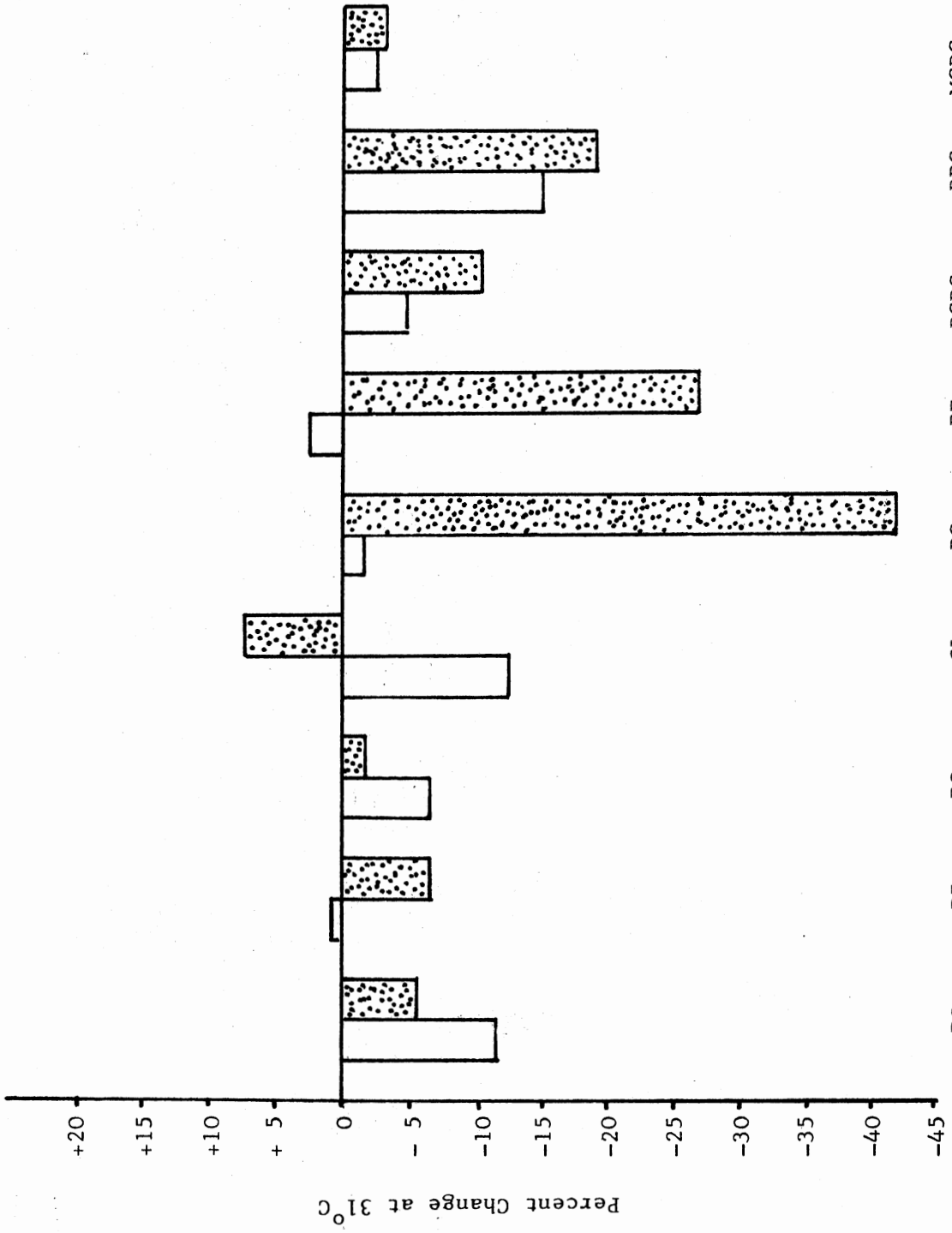

PS
PI
PC
SL
PG
PE
DGDG
DPG
MGDG
+30
+25
+20
+15
+10
+ 5
0
- 5
-10
-15
-20
-25
-30
Percent Change at 31°C

Figure 16. Comparison of the Changes in the Percent Dienoic Plus Trienoic Fatty Acids in Wheat and Milo Chloroplast Lipids When the Growth Temperature is Increased From 20°C to 31°C. Wheat is shaded.


PS
PI
PC
SL
PG
PE
DGDG
DPG
MGDG
+20
+15
+10
+ 5
0
- 5
-10
-15
-20
-25
-30
-35
-40
-45
Percent Change at 31°C

(1) that SL is more important in conferring thermal stability of the membrane protein boundary lipids based on the increased saturated/unsaturated values of milo over wheat which decreases;

(2) decreased levels of trienoic acids are more important in conferring thermal stability than decreased dienoic acids. Milo appears to be more heat tolerant because it seems to adjust to increased temperatures by decreasing the trienoic acid content of both SL and PG while dienoic fatty acids increase in both SL and PG. Wheat adapts to higher temperatures by large decreases in the dienoic content of PG with slight increases in SL. The slight difference observed between the trienoic fatty acids of PG from wheat (-16%) and milo (-17%) suggests that chloroplast PG would have little effect on the apparent difference in thermal tolerance of these two plants. These observations place greater importance on the trienoic acid changes in wheat and milo chloroplast SL. Although these changes are small in comparison to PG (-6% in milo and +1% in wheat), they may be very important due to the significant decrease observed in milo SL.

The second possibility relating thermal tolerance to the stability of the membrane fluid bilayer is facilitated by adding the assumption that Anderson (1975) was correct in identifying galactolipids as the major lipids of the chloroplast membrane fluid bilayer.

The saturated/unsaturated fatty acids of MGDG show the expected increase at increased growth temperature in milo (+8%) while an

unexpected decrease occurred in wheat (-2%). DGDG exhibited changes in saturated/unsaturated fatty acid ratios just opposite of those of MGDG with wheat increasing as expected (+12%) and milo decreasing (-4%). This data suggests that MGDG is more important in conferring thermal tolerance than DGDG.

Further analysis showed significant decreases in dienoic acid of MGDG with increasing temperature in wheat (-6%) while milo showed an increase of +1%. However, DGDG registered decreased levels of dienoic acids in milo (-5%) with insignificant temperature induced alterations in wheat (less than +1%).

Trienoic fatty acids unexpectedly increased in MGDG of wheat (+8%) chloroplast but decreased (-4%) in MGDG of milo at higher growth temperatures as expected. Again, just the reverse was true for DGDG. Trienoic acids of wheat DGDG decreased (-11%) as expected but increased less than 1% in milo (this may be insignificant).

Assuming milo is more heat tolerant than wheat as previously noted, this data suggests that trienoic fatty acids are again more important in conferring thermal stability than dienoic acids but fails to adequately pinpoint which galactolipid is most important in conferring thermal stability to the membrane lipid bilayer. The ratios of MGDG/DGDG further cloud the elucidation of the relationship of these lipids and membrane stability. Wheat showed increased MGDG/DGDG at increasing temperature (1.46 at 20^{o}C and 1.69 at 31^{o}C) while milo showed decreased ratios with increasing temperature 1.29 at 20^{o}C and 1.15 at 31^{o}C).

The possible functions of the other lipids are unknown. All except PC (5-7%) appear in very small quantities and may actually be

contaminants, although there is no conclusive evidence to this effect.

These data support the conclusion of Bjorkman (1975) conferring major significance to the protein boundary lipids SL and PG in conferring thermal stability to chloroplast membranes primarily by decreasing the trienoic acid composition of SL.

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VITA[2]

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